Matrix Metalloproteinase Activation and Inhibition

Thesis

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Matrix Metalloproteinase Activation and Inhibition

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Abstract

Processes leading to the *in vitro* activation of progelatinase-A, progelatinase-B and procollagenase were examined. Progelatinase-A can be activated by treatment with 4-aminophenyl mercuric acetate or by matrilysin and collagenase. The former induces an intramolecular self-cleavage before further intermolecular cleavages generate the mature enzyme. Whilst matrilysin can process progelatinase-A to the mature form, collagenase can only generate an intermediate by cleaving after N_{37} in the bait region. This intermediate is identical to that produced by a human tumour cell-line in culture. Gelatinase-A reciprocates the action of collagenase by completing the activation of the latter to generate a form of collagenase with full collagenolytic activity. This has been described as superactivation and is commonly associated with the action of stromelysin. It correlates with the appearance of F_{81} at the amino-terminus.

The C-terminal domain plays no role in catalysis but may be important for *in vivo* activity. This is particularly evident for collagenase which loses the ability to cleave collagen in its absence. The interaction between collagenase and collagen was shown to be a potential target for designing inhibitors of collagen breakdown.

The properties of a series of reversible, competitive hydroxamic acid matrix metalloproteinase inhibitors were studied. Incorporation of a phenylpropyl group in the P_1' position produced inhibitors with picomolar affinities for the gelatinases and discriminated against collagenase and matrilysin and other classes of zinc metallopeptidases. The high affinity of these inhibitors for gelatinase-A was shown to derive from a combination of diffusion limited association rates and very slow dissociation rates.

Methods were developed to allow the study of the pharmacokinetic properties of these inhibitors. Chemical modifications at the P_2' and P_3' positions were shown to result in inhibitors with oral bioavailabilities of greater than 30% in the mouse.

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I am indebted to Di Eaton for loaning me some of her Ki's and to Tom Crabbe for providing purified enzyme and for throwing in a few papers, all of which are probably quoted somewhere within the bowels of this thesis. Talking of which, *In vivo* studies were performed in collaboration with members of Ceiitech Oncology Pharmacology, notably, Pari Antoniw and Neville Willmott (pleural cavity assay) and Surinder Chander (pharmacokinetics).

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Abbreviations

ACE	Peptidyl-dipeptidase A
APMA	4-aminophenyl mercuric acetate
AP-N	Aminopeptidase-N
AU	Absorbance units
AUC	Area under the curve
BRIJ 35	Polyoxyethylene 23 lauryl ether
°C	degrees centigrade
CAPS	(3-[cyclohexylamino]-1-propanesulphonic acid
cDNA	Complementary deoxyribonucleic acid
CE	Capillary electrophoresis
CL	Collagenase
Con-A	Concanavalin-A
CPK	Corey Pauling Koltun
cpm	counts per minute
Da	Dalton
DCM	Dichloromethane
DMSO	Dimethylsulfoxide
Dnp-PLGLWAR	Dinitrophenyl Pro-Leu-Gly-Leu-Trp-Ala-(D)-ArgNH_2
Dpa	2,4-dinitrophenyl)-L-2,3-diaminopropionyl
ECL	enhanced chemiluminescence
EDTA	Ethylenediaminetetra-acetic acid disodium salt
ESAF	Endothelial cell stimulating angiogenic factor
[E]	enzyme concentration
E_t	Total enzyme concentration
E_z	Molar extinction coefficient at wavelength z nanometres
Fig.	Figure
FU	Fluorescence units
g	gram

GL-A	Gelatinase-A
GL-B	Gelatinase-B
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFC	Human fibroblast collagenase
HNC	Human neutrophil collagenase
hr(s)	hour(s)
HPLC	High pressure liquid chromatography
HRP	Horseradish peroxidase
Hyl	Hydroxylysine
Hyp	Hydroxyproline
[I]	inhibitor concentration
I_t	Total inhibitor concentration
IC_{50}	Inhibitor concentration producing 50% inhibition of enzymic activity
IgA	Immunoglobulin A
i.p.	intraperitoneally
i.v.	intravenous
kDa	kilodalton
k_{cat}	turnover number
Ki	inhibition constant
Ki_{app}	Apparent inhibition constant
K_m	Michaelis constant
k_{obs}	pseudo first order rate constant for the establishment of equilibrium between enzyme-inhibitor complexes
k_{off}	dissociation rate constant
k_{on}	association rate constant
kV	kilovolt
IL-1	Interleukin-1
λ	wavelength
λ_{em}	emission wavelength
λ_{ex}	excitation wavelength

LHS	Left-hand-side
L_n	natural logarithm
μ	micro
m	milli
M	Molar
Mca	Methoxycoumarin
ml	millilitre
MAb	Monoclonal antibody
mA	milliampere
min(s)	minutes(s)
MMP	Matrix metalloproteinase
mOD unit	milli optical density unit
MPI	Metalloproteinase inhibitor
mRNA	Messenger ribonucleic acid
MRT	mean residence time
MT-MMP	Membrane type matrix metalioproteinase
NEP	Neutral endopeptidase (neprilysln)
NGL-A	Gelatinase-A_1 - 417
nm	nanometre
NMR	Nuclear magnetic resonance
p	pico also para
PBS	Phosphate buffered saline
p.o.	per os (by mouth)
PMNL	Polymorphonuclear leucocyte
PMSF	Phenylmethyl sulphonyl fluoride
proNGL-A	Progelatinase-A_1 - 417
PZP	Pregnancy zone protein
QF24	7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-ArgNH_2
RHS	Right-hand-side
rpm	revolutions per minute

r/t	room temperature
S	substrate concentration
SAR	Structure-activity relationship
SBTI	Soya bean trypsin inhibitor
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec(s)	second(s)
$t1_{/2}$	half-life
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TIMP	Tissue inhibitor of metalloproteinases
TNF	Tumour necrosis factor
TPCK	*N* - tosyl-L-phenylalanine chloromethyl ketone
TRIS	(Tris [hydroxymethyl] amino-methane)
triton X-100	Iso-octylphenoxypolyethoxyethanol
UV	ultraviolet
V	volt
v_o	Initial steady state velocity
v_s	Final steady state velocity
V_D	volume of distribution
V_{max}	maximum velocity
v/v	volume per volume
w/v	weight per volume

Amino Acid Abbreviations

Ala (A)	Alanine
Arg (R)	Arginine
Asn (N)	Asparagine
Asp (D)	Aspartic acid
Cys (C)	Cysteine

Gln (Q)	Glutamine
Glu (E)	Glutamic acid
Gly (G)	Glycine
His (H)	Histidine
Ile (I)	Isoleucine
Leu (L)	Leucine
Lys (K)	Lysine
Met (M)	Methionine
Phe (F)	Phenylalanine
Pro (P)	Proline
Ser (S)	Serine
Thr (T)	Threonine
Trp (W)	Tryptophan
Tyr (Y)	Tyrosine
Val (V)	Valine

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Chapter 1

CHAPTER 1
INTRODUCTION

1.1: Introduction

In cancer the spread of tumour cells from a primary site to colonise distant organs is called metastasis. When cancer treatment fails it is the primary cause of patient death (Liotta, 1992). Proteolytic enzymes belonging to the matrix metalloproteinase (MMP) family have been implicated in this process and are currently the target of a large pharmaceutical effort to develop novel anti-metastatic agents. The work presented in this thesis examines (1) The biochemistry of these enzymes and (2) the design of synthetic inhibitors of MMPs. An overview of this field of research is presented in this Chapter in order to provide a basis for discussion in later Chapters.

1.2: Tumour invasion and metastasis

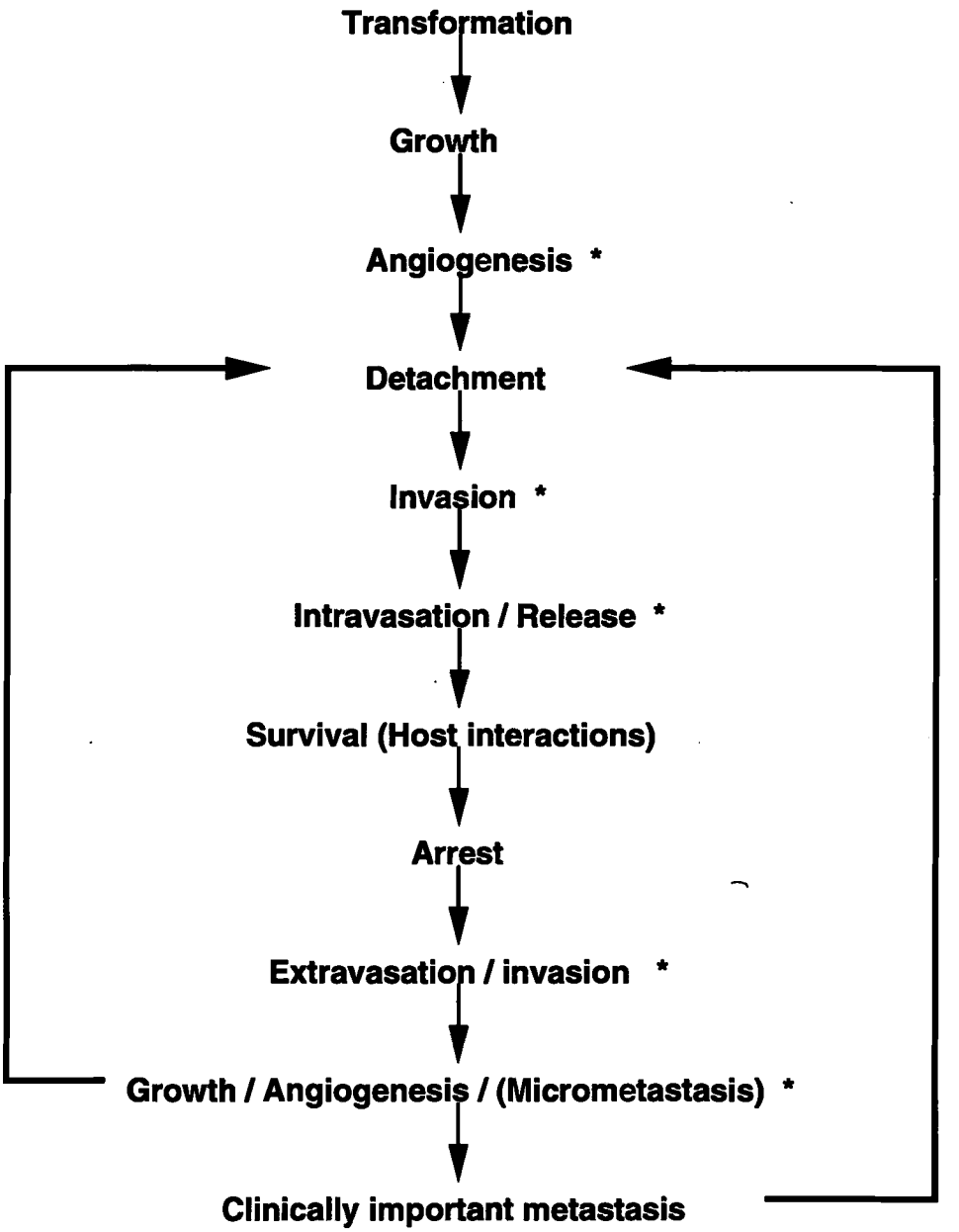
1.2:1 The stages of tumour invasion and metastasis

Basement membranes play an important role in maintaining the integrity of organ structures in the body. The destruction of these membranes is a common feature of tumour growth. They are comprised of high molecular weight matrix proteins namely, type IV collagen, laminin and proteoglycans together with small amounts of nidogen (Kleinman *et al.*, 1982; Hassell *et al.*, 1985). The surrounding stroma is also made up of matrix molecules, of which the fibrillar collagens are the major structural elements. Tumour invasion is the process by which tumour cells disrupt these anatomical barriers and invade the surrounding tissues. Some tumour cells also acquire the capability to enter the lymphatic or blood system (intravasate) and then to escape the circulation (extravasate) and proliferate at a secondary site. This is known as metastasis and it depends on a sequence of events, some of which allow the cells to attach to basement membranes and others which allow the cells to penetrate these membranes and invade the underlying tissue (Fig.1.1). Endopeptidases have been implicated in stages which require the dissolution of physical barriers. Additionally, since the formation of new blood vessels (angiogenesis) requires the penetration of

Figure 1.1

The metastatic cascade

The steps involved in tumour cell invasion and metastasis



Tumour cell invasion and metastasis is a sequential process involving many cell:cell and cell:matrix interactions.The above diagram (Hart and Saini, 1992) illustrates the steps required for a tumour cell to metastasise from a primary tumour to a secondary site. Steps where proteinases are presumed to be involved are labelled (*)

basement membrane by endothelial cells these same enzymes may contribute to the growth of the tumour itself (reviewed in Liotta *et al.*, 1991).

1.2:2 The involvement of proteinases in tumour invasion and metastasis

There are four major classes of proteinase which can be categorised according to the amino acid residues involved in catalysis. Elevated levels of all of these classes (aspartyl, serine, cysteine and metallo) have been correlated with tumour invasiveness (reviewed in Moscatelli and Rifkin,1988; Duffy, 1992). These include plasminogen activators (serine) (Quigley *et al.*, 1987; Reich *et al.*, 1988; Dano *et al.*, 1985; Schmitt *et al.*, 1992), cathepsin B (cysteine), cathepsin D (aspartyl) (Thorgeirsson *et al.*, 1985; Rochefort, 1990; Sloane *et al.*, 1990; Chen, 1992) and a range of matrix metalloproteinases (Liotta *et al.*, 1980; Ostrowski *et al.*, 1988; Templeton *et al.*, 1990; McDonnell and Matrisian, 1990; Stetler-Stevenson, 1990).

Increased MMP expression has been correlated with the invasiveness of several tumour types and the down regulation of these enzymes reduces the invasive properties of manipulated cell lines (Goldberg and Eisen, 1992a). Moreover, transfection of the cDNAs encoding several members of this family into non-metastatic cell-lines conveyed a metastatic phenotype to these cells (Cockett, 1993; Bernhard *et al.*; 1994; Powell *et al.*, 1993). A matrix metalloproteinase inhibitor has been reported to inhibit the lung colonisation of B16 F10 melanoma cells in a syngeneic mouse recipient (Reich *et al.*, 1988) and recently, a selective inhibitor of gelatinase-A has been shown to block lung colonisation by COLO 26 cells (Cockett, 1993).

This thesis provides the background to the design and characterisation of such inhibitors, together with a biochemical characterisation of gelatinase-A and B and interstitial collagenase.

1.3: The matrix metalloproteinases

1.3:1 General properties of matrix metalloproteinases

The matrix metalloproteinases (MMPs) are thought to orchestrate the normal tissue remodelling associated with cell migration, angiogenesis, tissue morphogenesis and growth, uterine involution and cervical softening (reviewed

in Matrisian, 1990). Breakdown of the tight regulation of these enzymes can lead to the inappropriate matrix modelling observed in diseases such as the arthritides, fibrosis, atherosclerosis, multiple sclerosis and tumour invasion and metastasis (Murphy and Reynolds, 1993; Stetler-Stevenson, 1990).

The MMPs are comprised of a multi-gene family of zinc and calcium dependent proteinases which are capable of hydrolysing all the major elements of both basement membrane and connective tissue matrix at physiological pH (Docherty *et al.*; 1992; Murphy and Reynolds, 1993) (Table 1.1). They are modular enzymes comprised of distinct homologous domains two of which, the propeptide and the catalytic, are common to the entire family.

MMPs typically hydrolyse the bond between a small neutral amino acid residue such as glycine or alanine and a large hydrophobic residue such as leucine or phenylalanine. They are secreted as latent proenzymes by connective tissue cells as well as by cells of haematopoetic origin. Latency is maintained by the presence of an approximately 80 amino acid N-terminal propeptide which is removed upon activation. This can be initiated *in vitro* by the addition of the organomercurial, aminophenylmercuric acetate (APMA) in a process leading to the auto-catalytic cleavage of the propeptide (Nagase *et al.*, 1991). The susceptibility of MMPs to organomercurial initiated activation is a distinguishing feature of this family (section 1.3:3).

Once activated the enzymes are thought to be regulated by inhibitors such as the general proteinase inhibitor α-2 macroglobulin (α-$_2$M) and also by a family of MMP specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) (section 1.4:2) (Cawston, 1986). Although there is a high degree of amino acid sequence homology across the family (reviewed in Birkedal-Hansen, 1993), they can be conveniently subdivided into three major classes on the basis of structure and function.

(i) The collagenases

There are three known members of the collagenase family, interstitial collagenase (also known as fibroblast collagenase), neutrophil collagenase and a cDNA coding for a third member of the family has recently been cloned from a breast tumour derived cDNA library (Freije *et al.*, 1994). Collagenases are the most specialised of the known MMPs. Their ability to cleave fibrillar collagen

Table 1.1

The human matrix metalloproteinase family

ENZYME	E.C. NO.	MOLECULAR MASS (kDa)		MATRIX SUBSTRATES
		Latent	Active	
Interstitial collagenase	3.4.24.7	55	43	collagen types I, II, III, VII and X, gelatin (limited) proteoglycan core protein
Neutrophil collagenase	3.4.24.34	75	58	collagen types I, II, III, gelatin (limited) proteoglycan core protein
Collagenase-3	—	65	55	type I collagen
Gelatinase A	3.4.24.24	72	66	denatured collagens, collagen types IV, V,VII, XI
Gelatinase B	3.4.24.35	92	86	denatured collagens, collagen types IV and V, elastin
Stromelysin 1	3.4.24.17	57	46	proteoglycan core protein, collagen types II' IV, IX, X and X1, procollagen, fibronectin, laminin, elastin (poor), gelatin (limited)
Stromelysin 2	3.4.24.24	57	46	
Matrilysin	3.4.24.23	28	20	proteoglycan, type IV collagen, elastin, fibronectin and gelatin
Macrophage Elastase	—	54	45 (22)	elastin
Stromelysin 3	—	51	44	unknown
MT-MMP	—	66	—	progelatinase-A

Molecular masses are for the human enzymes and are estimated by SDS-PAGE (reducing conditions) except for MT-MMP (Sato et al., 1994) which is predicted from the cDNA. Macrophage elastase (Shapiro et al., 1993) spontaneously activates in the absence of EDTA to a 22 kDa form which lacks the C-terminal domain.

(mainly types I ,II and III) at a single locus within the triple helical domain makes them unique amongst mammalian proteinases. Under most circumstances very little of the mesenchymal derived enzyme (interstitial collagenase) is synthesised but mRNA levels can be significantly increased in response to various cytokines and growth factors. The up-regulation of the enzyme in response to interleukin-1 (IL-1) and tumour necrosis factor (TNF) is of particular interest because of the contribution of these cytokines to the progression of inflammatory disorders such as rheumatoid arthritis.

A closely related enzyme is synthesised by neutrophils. Unlike the fibroblast form it is stored in granules prior to its release in response to neutrophil activating agents. The latent and active forms of neutrophil collagenase are somewhat higher in molecular mass than their fibroblast counterparts but this is largely a result of glycosylation. The two enzymes exhibit similar substrate specificities but the neutrophil enzyme is catalytically more efficient and cleavage at aryl amino acids occurs more readily (Netzel-Arnett *et al.,* 1991).

The third member contains several residues specific to the collagenases as well as the shortened proline-rich hinge region which is also a characteristic of this family. The expressed protein justifies its classification as a collagenase by its ability to degrade fibrillar collagen.

(ii) The stromelysins

Stromelysins-1 and 2 are secreted from mesenchymal cells as 57 kDa proenzymes. Comparison of the enzymic properties of the two enzymes (Nicholson *et al.,* 1989) suggest that they have almost identical substrate specificities. The production of stromelysin-1 by rheumatoid synovial hypertrophic lining cells but not normal synovial cells (Okada *et al.,* 1989a) and the elevated level of proteoglycan-degrading activity found in osteoarthritic cartilage compared with normal cartilage (Martel-Pelletier *et al.,* 1984; Dean *et al.,* 1989), indicate that these enzymes play an important role in the destruction of cartilage. The stromelysin-1 gene shows similarities in the promoter/enhancer region to interstitial collagenase and consequently is often co-ordinately produced upon stimulation with growth factors or tumour promoters (Frisch *et al.,* 1987; Edwards *et al.,* 1987). The stromelysins possess the broadest substrate specificity (Table 1.1) and may play an additional physiological role in the activation of other members of the family (Ogata *et al.,* 1992; O'Connell *et al.,* 1994; Murphy *et al.,*

1987). Stromelysin-1 is active on a low molecular mass synthetic substrate between pH 4 and pH 10 (Harrison *et al.*, 1992) but it has a very narrow pH optimum of pH 5 - 5.5.

(iii) The gelatinases

Harris and Krane (1972) first described a metalloproteinase released by cultured rheumatoid synovium that was capable of degrading denatured interstitial collagen (gelatin). The enzyme was subsequently found to be identical to a 72 kDa type IV collagenase isolated from cultures of rabbit bone calvaria by Murphy *et al.* (1985). This enzyme (gelatinase-A) is constituitively expressed by a number of normal and transformed cells (Seltzer *et al.*, 1981; Salo *et al.*, 1991; Lefebvre *et al.*, 1991; Overall and Sodek, 1987) and is the most widely distributed of all the MMPs. Transcriptional control of gelatinase-A is different to that of collagenase and stromelysin. Low levels of the enzyme are expressed by most cells and these levels are not regulated by agents such as IL-1 and TNF (reviewed in Matrisian,1990).

Gelatinase-A is effective against type V and type VII collagens (Seltzer *et al.*, 1989) in addition to type IV collagen, the major structural component of basement membrane. It may also act co-ordinately with collagenase to complete the degradation of fibrillar collagen and thus aid in its elimination from the body.

Progelatinase-B is a 92 kDa enzyme that was originally recognised as a gelatinolytic metalloproteinase of PMN leukocytes by Sopata and Dancewicz (1974). Whilst it is secreted by many cell types in culture the same gene product is produced in a storable form in PMNs. Although the two gelatinases share structural homology and substrate specificity they differ at the transcriptional level since the gelatinase-B gene possesses elements in common with the stromelysins and interstitial collagenase (reviewed in Birkedal-Hansen *et al.*, 1993).

(iv) Other MMPs

Matrilysin

Matrilysin is the smallest member of the MMPs consisting of a catalytic and a propeptide domain only. It is expressed in developing monocytes (Busiek *et al.*, 1992) and gingival fibroblasts (Overall and Sodek, 1990) as a 28 kDa proenzyme which reduces to 20 kDa upon activation. Like the stromelysins, with

which it is sometimes classified it has a broad substrate specificity but it is distinguishable from the former by its broad pH optimum (pH 6 - 8) (Crabbe *et al.*, 1992).

Metalloelastase

A metalloelastase, sharing about 50% homolgy with collagenase and stromelysin, has been identified in human alveolar macrophages (Shapiro *et al.*, 1993). The deduced amino acid sequence predicts a 54 kDa proenzyme. In the absence of EDTA, this readily undergoes amino- and carboxy-terminal processing to a mature 22 kDa form. Partially purified native metalloelastase degrades insoluble elastin.

Stromelysin-3

Stromelysin-3 was first identified in the stromal cells surrounding invasive breast carcinomas by Basset *et al.* (1990). Its name is rather misleading since it shares no greater sequence homology with stromelysin than with collagenase and on this basis cannot be classified with either group. To date, insufficient quantities of the active human enzyme have been isolated to enable its substrate specificity to be determined although the mouse homologue is reported to degrade laminin and fibronectin (Murphy *et al*, 1993).

MT-MMP

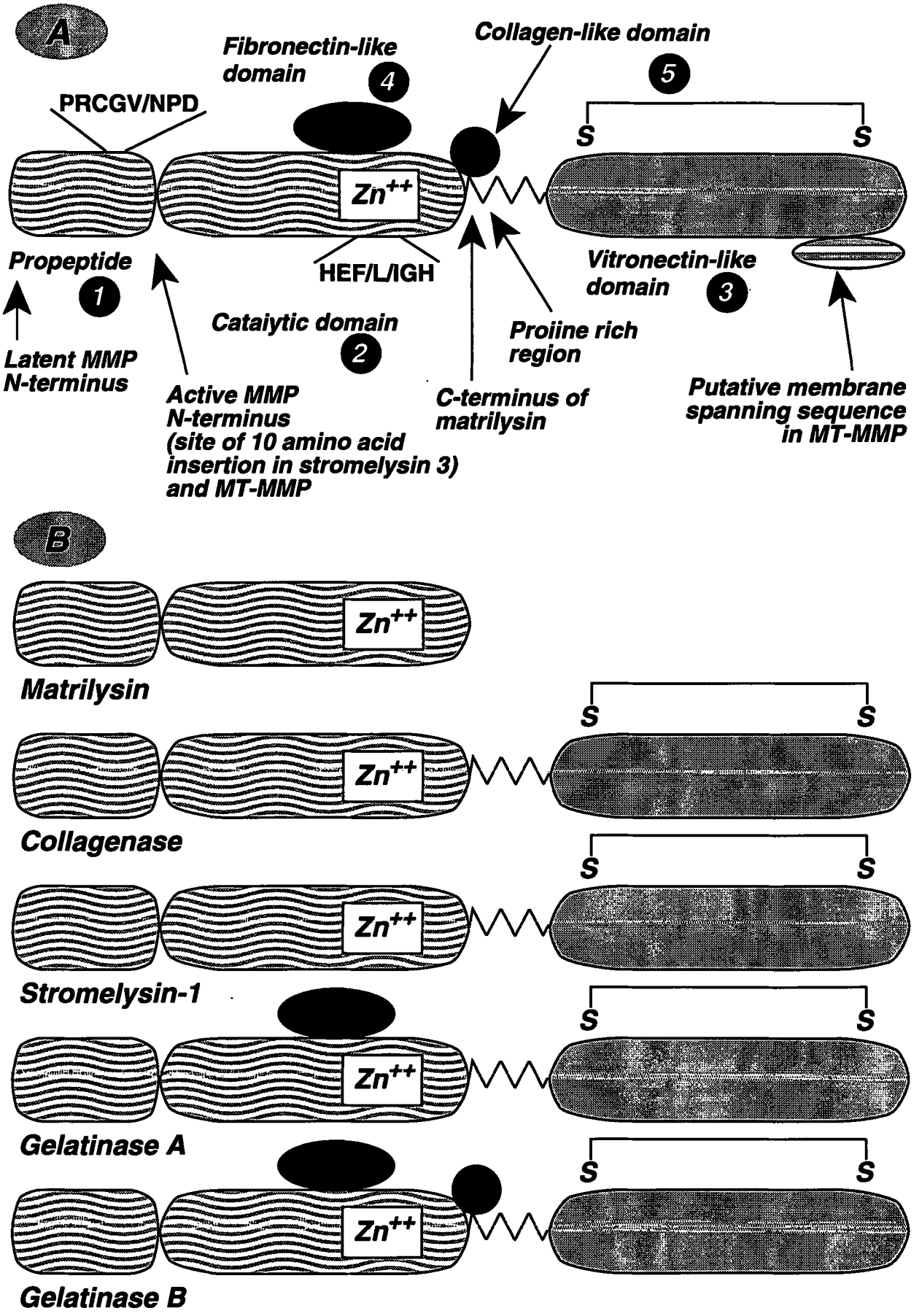
Stromelysin-3 shares a region of homology located between the end of the propeptide and the start of the catalytic domain with a membrane bound MMP (MT-MMP) recently isolated from a human placental cDNA library (Sato *et al.*,1994). The latter enzyme was shown to be expressed in lung carcinomas and to a lesser extent in normal lung. Its most striking feature is the presence of a hydrophobic tail which may be involved in anchoring it to the cell membrane. Its only known substrate to date is progelatinase-A

1.3:2 The structure and function of MMP domains

Inspection of MMP structures reveals that they can be divided into discrete functional domains (Fig. 1.2).

Figure 1.2

The domain structures of matrix metalloproteinases



Domain 1 (Propeptide)

Domain 1 comprises the first 80 amino acid residues or so of the secreted enzyme. After secretion the main function of this peptide is to maintain the enzyme in a latent form. This is discussed in detail in section 1.3:3. In common with the propeptide of other enzymes (Zhu *et al.*, 1989) it may also have a role in promoting the intracellular folding of these enzymes.

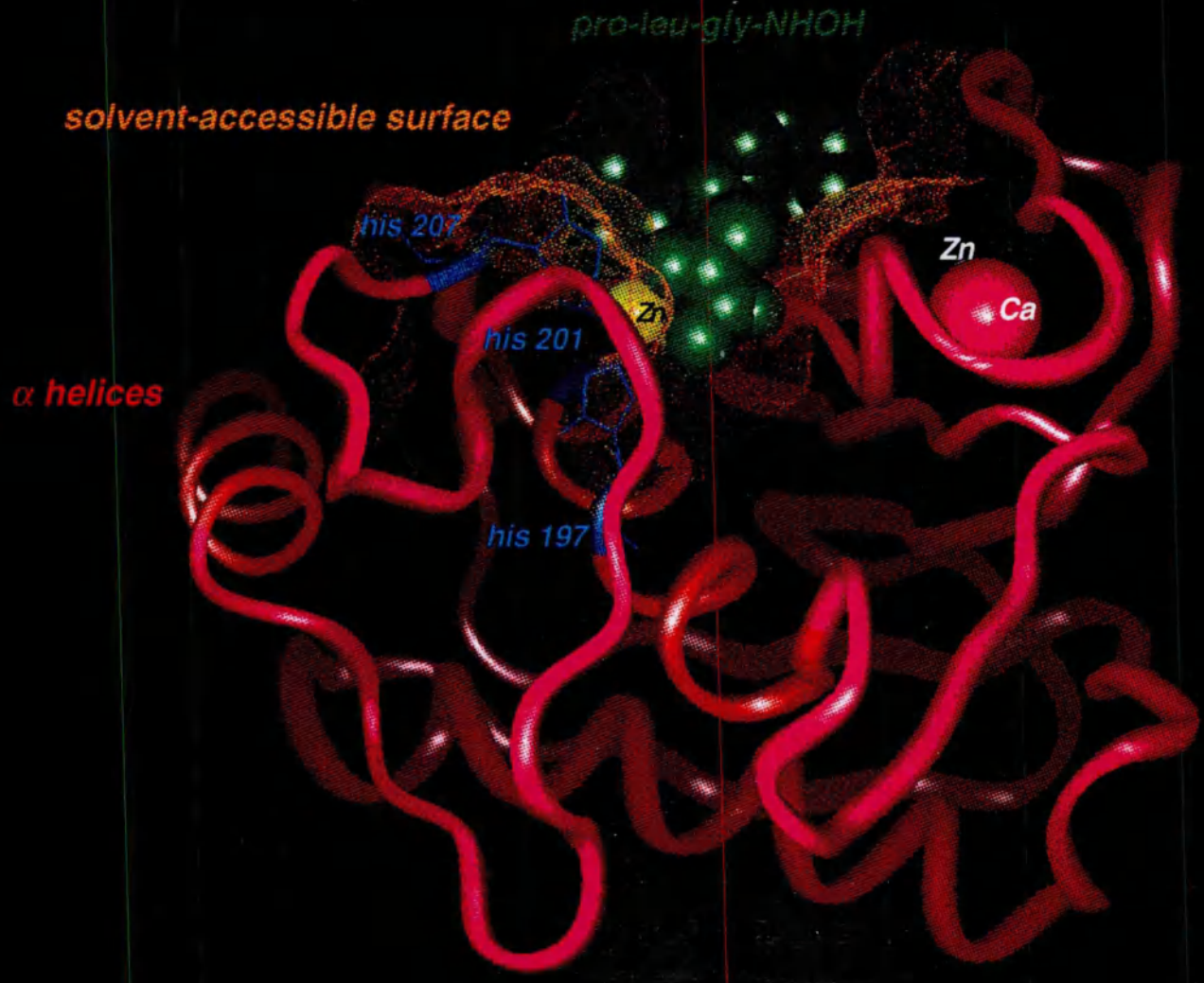
Domain 2 (Catalytic domain)

Domain 2 possesses the catalytic machinery including the substrate binding cleft and the active site. This domain can be delineated both genetically and functionally from the hinge region of domain 3 by a (L/I) Y G amino acid sequence (L^{260} in human interstitial collagenase). Towards its C-terminus is a sequence which shares very strong homology with the sequence Val^{139}-V-A-H^{*}-**E**-L-T-H^{*}-A found in the bacterial endopeptidase thermolysin. This 'zinc box' is the signature for zinc endopeptidases and includes two histidines (H^{*}) which bind to the catalytic zinc. MMPs differ from thermolysin in that the third zinc binding residue is also a histidine as opposed to a glutamic acid in the bacterial enzyme. The catalytic properties of glutamic acid (**E**) in the above sequence, previously described for thermolysin (Matthews *et al.*, 1988), have been confirmed in gelatinase-A by the observed reduction in activity resulting from its replacement by A, Q or D (Crabbe *et al.*, 1994a).

The structure of this domain has now been solved by X-ray crystallography (Bode *et al.*, 1994; Lovejoy *et al.*, 1994; Stams *et al.*, 1994; Borkakoti *et al.*, 1994; Reinemar *et al.*, 1994; Spurlino *et al.*, 1994) and NMR (Gooley *et al.*, 1994). The crystal structure of the neutrophil collagenase catalytic domain showing the substrate binding cleft and a bound inhibitor is presented in Figure 1.3. This structure is representative of the MMP structures described to date. The crystal structure reveals a spherical molecule with a shallow active site cleft. The cleft separates a small C-terminal region from a larger N-terminal region composed of two α-helices, a five-stranded β-sheet and bridging loops. The catalytic zinc is situated at the bottom of the cleft and is co-ordinated by three histidines in the sequence **His**^{197}- E-F-G-**H**-S-L-**G**-L-A-**H**. An important feature of these enzymes is the presence of a conserved glycine (**G**) (above) which plays an important role in enabling the third histidine to bind to the catalytic zinc. This places the MMPs in the same group as astacin (Bode *et al.*, 1992) and the snake

Figure 1.3

The structure of the catalytic domain of human neutrophil collagenase, complexed to pro-leu-gly-NHOH



Based on Bode et al (1994) (Insight II, Biosym Programme)

venom metallopeptidase, adamalysin II (Gomis-Ruth*et al.,* 1993). The name 'metzincin' has been coined by Bode *et al.* (1993) to describe this super-family. Besides the catalytic zinc, neutrophil collagenase has an additional zinc (bound by three histidine residues and an aspartic acid) which together with two calcium ions is thought to play a structural role. Only one calcium has been identified in other MMPs (Spurlino *et al.,* 1994). The conserved calcium appears to make contacts with residues on either side of the zinc box supporting the view that it plays an important structural role. The residues involved in substrate and inhibitor recognition are discussed in section 1.4:4 (ii).

Domain 3 (C-terminal / hemopexin / vitronectin-like domain)

Domain 3 is present in all the MMPs with the exception of matrilysin and is separated from the catalytic domain by a proline-rich hinge of varying length. It contains regions of homology with both hemopexin and vitronectin and in gelatinase-A alone, there is a sequence of significant homology with the immunoglobulin A (IgA) proteinases (unpublished observation). Two conserved cysteines can be used to align the primary sequences of this domain for all the MMPs (Fig. 1.2). The presence of an intact disulphide bridge between these cysteines may be important for maintaining the structure of the domain.

For most of the MMPs the C-terminal domain has been shown to play no role in catalysis since domain 2 retains its activity on low molecular mass substrates in its absence (Sanchez-Lopez *et al.,* 1993; Lowry *et al.,* 1992; Murphy *et al.,* 1992a). It does, however, possess some important non-catalytic properties. Several groups have shown that the C-terminal domain of interstitial collagenase (Birkedal-Hansen *et al.,* 1988; Clark and Cawston, 1989; Murphy *et al.,* 1992b) and neutrophil collagenase (Schnierer *et al.,* 1993) is essential for the cleavage of fibrillar collagen although it plays no role in the activity of these enzymes against peptide substrates. Both collagenase and stromelysin have been shown to bind to collagen through their C-terminal domains. Interestingly, the presence of the propeptide inhibits this interaction in collagenase but not stromelysin (Murphy *et al.,* 1992b).

The C-terminal domain of gelatinase-A has been shown to be dispensable for catalysis and substrate recognition but it is necessary for activation by cell membranes (Murphy *et al.,* 1992a). This work has been extended by Cockett (1993) who has demonstrated that cells transfected with gelatinase-A cDNA have

a metastatic phenotype that depends on the expressed gelatinase-A possessing an intact C-terminal domain.

The ability of progelatinase-A and B to bind to TIMP-2 and TIMP-1 respectively, has also been localised to their C-terminal domains (Goldberg *et al.*, 1989). Additionally, the presence of this domain increases the strength of TIMP binding to the active enzymes (Howard *et al.*, 1991; Murphy *et al.*, 1992a). Goldberg *et al.* (1992b) have suggested that the domain may contribute to the activation of progelatinase-B by stromelysin but this is not substantiated by the results of other groups (O'Connell *et al.*, 1994). These studies are described in Chapter 3.

Domain 4 (gelatin-binding)

Domain 4 is formed by three repeating motifs which share homology with the gelatin binding region of fibronectin. It is only present in the two gelatinases and may mediate the sequestration of these enzymes within the extracellular matrix (Collier *et al.*, 1988; Wilhelm *et al.*, 1989). It is inserted into a conserved region of amino acids just prior to the active site and may form a discrete domain which juts out from the main body of the enzyme. It is required for both binding to type I collagen and to confer specificity for type IV collagen (Murphy *et al.*, 1994)

Domain 5 (collagen-like)

Domain 5 is found in gelatinase-B alone and may simply be an extension of the proline hinge region. It shares a weak homology with some types of collagen (Wilhelm *et al.*, 1989) but its function is unknown.

Other motifs

Pro/active domain insertion

Stromelysin-3 and MT-MMP possess a 10 amino acid insertion situated between the conserved P-R-C-G-(V/N)-P-D sequence of the propeptide and the N-terminus of the catalytic domain. Interestingly, it contains the motif, R-X-K-R, which is the substrate recognition sequence for subtilisin-like processing enzymes (reviewed in Smeekens, 1993): Hosaka *et al.*, 1991). Since these enzymes act within the cell it is possible that stromelysin-3 and MT-MMP are processed intracellularly and then secreted (or inserted into the membrane) in an active form.

Membrane spanning domain

This is present in MT-MMP only and consists of 24 uncharged hydrophobic residues situated at the end of the C-terminal domain. By analogy with such motifs in other proteins (Sato *et al.*, 1994) it is presumed to anchor the enzyme to the cell membrane.

1.3:3 Activation of MMPs

The activation of MMPs represents a critical point in controlling their activity *in vivo*. The end-point of this process is the removal of the approximately 80 amino acid propeptide with a concomitant reduction in molecular mass of about 10 kDa on SDS-polyacrylamide gels.

The latency of the proenzyme is thought to be maintained by the insertion of a conserved sequence of the propeptide, P-R-**C**-G-(V/N)-P-D, into the active site cleft. The unpaired propeptide cysteine (C^{73} in interstitial collagenase) then acts as the fourth ligand for the catalytic zinc and displaces a catalytically essential water molecule (see section 1.3:4). Theoretically, the propeptide could be removed by a single cleavage downstream of the conserved cysteine. The fact that this does not occur, suggests that the susceptible bond which marks the beginning of the catalytic domain in the active form of the enzyme is masked by other regions of the propeptide (Nagase *et al.*, 1991).

Activation is a consequence of the displacement of the cysteine by a water molecule in a process that has been called the 'cysteine switch' (Springman *et al.*, 1990). There are a number of ways of achieving this *in vitro* but mechanistically these may be divided into two categories (1) chemical and (2) enzymic.

The first category includes organomercurials such as APMA, metal ions, reducing agents, SDS, heat and oxidants. They either chemically alter the cysteine (eg. APMA) or perturb the structure of the catalytic site sufficiently to allow water to enter and displace the cysteine from the zinc (Fig. 1.4 {A}). The catalytically competent enzyme then performs an intramolecular cleavage at a site 5 - 8 residues upstream of the propeptide cysteine which presumably lies in the substrate recognition cleft (Stricklin *et al.*, 1983; Grant *et al.*, 1987; Nagase *et al.*, 1990). An intermolecular, concentration dependent autolytic cleavage completes the process by removing C^{73} to form the mature enzyme. Whilst chemical activators (oxidants) have been reported to activate PMN derived

Figure 1.4

Chemical activation of matrix metalloproteinases

A

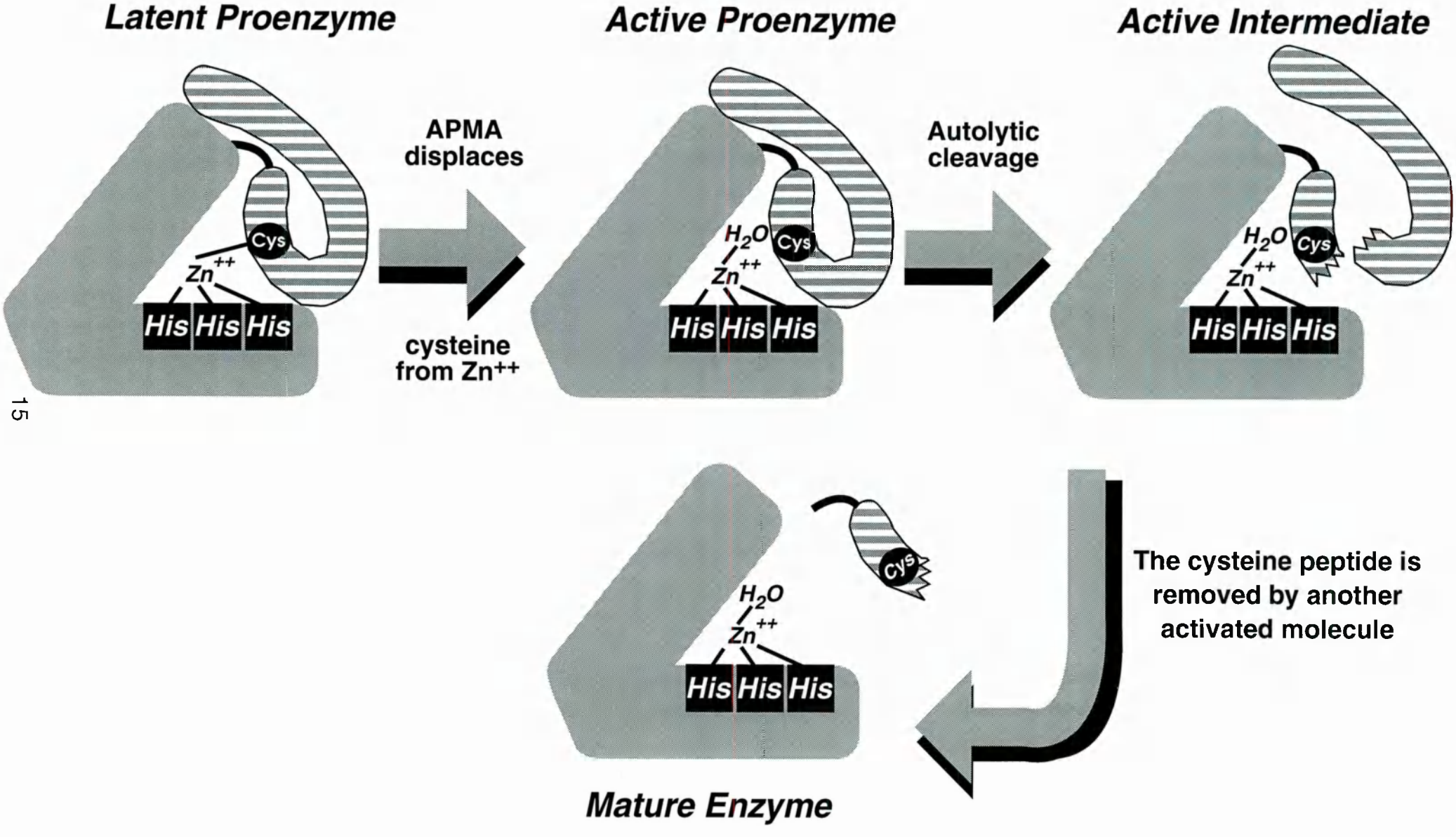
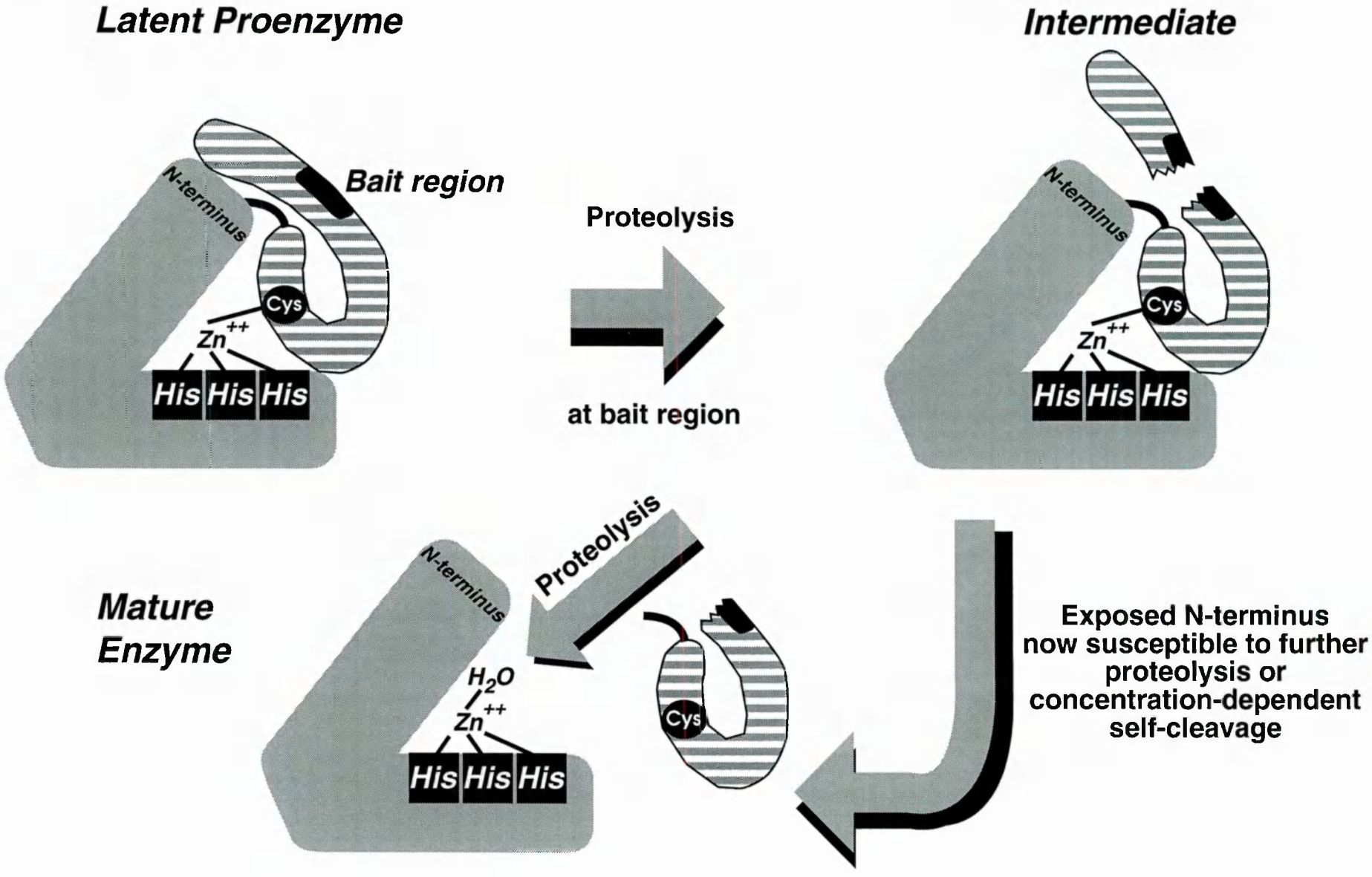


Figure 1.4

Proteinase activation of matrix metalloproteinases

B



enzymes *in vivo* (Weiss *et al.,* 1985: Burkhardt *et al.,* 1986) a physiological counterpart to APMA has not yet been isolated. A low molecular mass activator which might fulfil this role is endothelial cell stimulating angiogenic factor (ESAF) which has been reported to activate collagenase and gelatinase-A (Weiss *et al.,* 1983).

Removal of the propeptide domain may also be accomplished by the action of proteinases (Fig. 1.4 {B}). The archetypal serine proteinase, trypsin is routinely used for the test-tube activation of the collagenases, matrilysin and the stromelysins. The physiological counterparts to trypsin are presumed to be plasmin and kallikrein (Werb *et al.,* 1977). Proteinases make an initial cleavage in the propeptide at a point located 30-40 residues downstream from the N-terminus. This has been termed the 'bait' region although its role is not strictly analogous to the role of the bait region in α-2 macroglobulin (Barrett and Starkey, 1973). This proteinase sensitive region has an amino acid sequence which dictates the class of proteinase able to activate the MMP in question. Thus, the procollagenase bait region, Lys^{34}-R-R-N , can be cleaved by enzymes with trypsin-like specificity for basic residues. The stromelysin sequence of Phe^{34}-V-R-R-K may in addition be cleaved at the phenylalanine by enzymes with chymotrypsin specificity (Nagase *et al.,* 1990; Suzuki *et al.,* 1990). Of particular interest, since the MMPs are often co-ordinately expressed is the activation of one by another. Whilst the so-called 'superactivation' of interstitial procollagenase by stromelysin (Chapter 4) proceeds efficiently only on the partially processed enzyme (Murphy *et al.,* 1987) stromelysin activation of progelatinase-B is carried out directly by stepwise removal of the propeptide (Ogata *et al.,* 1992; O'Connell *et al.,* 1994). Similarly, both matrilysin and collagenase have recently been shown to activate progelatinase-A by cleaving a sequence which may be a bait region for MMPs (Crabbe *et al.,* 1994b; Crabbe *et al.,* 1994c) (Chapter 3). The activation of gelatinase-A by these two MMPs has so far only been demonstrated *in vitro* but the MT-MMP recently described by Sato *et al.* (1994) may assume this role *in vivo.* The enzyme is structurally related to the soluble MMPs and is probably responsible for the well documented activation of progelatinase-A by tumour cells (Brown *et al.,* 1993; Azzam *et al.,* 1993) or concanavalin-A stimulated fibroblasts (Ward *et al.,* 1991).

1.3:4 The mechanism of action of MMPs

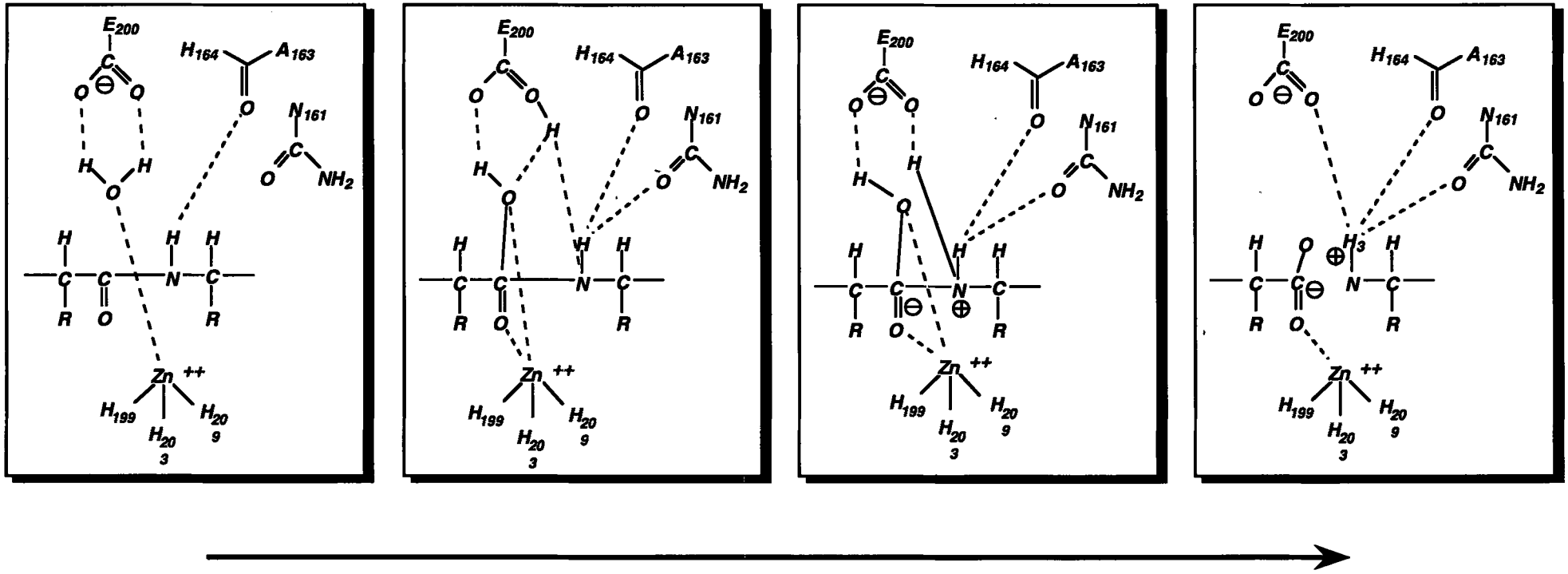
In order to create the correct chemical environment for hydrolytic attack, proteinases have developed a ubiquitous catalytic apparatus. The basic elements of this apparatus are a nucleophile, a general base and an electrophile. For the MMPs these are water, glutamic acid and zinc, respectively. A mechanism for MMP catalysed substrate cleavage can be proposed by considering the structural evidence provided by X-ray crystallography. Spurlino *et al.* (1994) and Lovejoy *et al.* (1994b) have postulated that many features of the interstitial collagenase catalytic mechanism (Fig. 1.5) are consistent with the mechanism proposed by Matthews (1988) for the action of the structurally related bacterial zinc proteinase, thermolysin. In the free enzyme the zinc ion is co-ordinated to the side chains of H^{199}, H^{203}, H^{209} and a water molecule (residue numbers refer to the proenzyme form). Cleavage is initiated by the nucleophilic attack of water on the scissile peptide bond. The attack is promoted by E^{200} situated in the zinc box sequence Val^{196}-A-A-H-**E**-L-G-H. The carbonyl oxygen of A^{163} and the side-chain oxygen of N^{161} then stabilize the protonated nitrogen of the scissile bond. In contrast to thermolysin the carbonyl oxygen of the cleaved bond appears to be stabilised by the carbonyl-zinc interaction alone. A tetrahedral intermediate is formed from the attack of the activated water molecule on the carbonyl group of the scissile bond. The subsequent collapse of this intermediate then gives a primary ammonium species and a zinc bound carboxylate. Release of the protonated amine and expulsion of the product carboxylic acid permit the water molecule to co-ordinate to the zinc to complete the cycle.

1.3:5 Cleavage-site specificity

As noted earlier the MMPs often hydrolyse bonds between small uncharged amino acids and large hydrophobic residues. In designing inhibitors, however, it is possible to take advantage of subtle differences in selectivity between members of the family. A selection of cleavage site sequences are presented in Figure 1.6. These are representative of the preferred substrate cleavage sites of various MMPs (reviewed in Birkedal-Hansen *et al.,* 1993). By convention amino acids to the left of the cleavage site are designated P_1, P_2, P_3 etc. and interact with corresponding sub-sites on the enzyme S_1, S_2, S_3 and

Figure 1.5

A proposed mechanism for the cleavage of peptides by fibroblast collagenase



Taken from Spurlino et al (1994). Amino acids are numbered from the N-terminus, ^{1}F P A T L^{5} of the proenzyme

Figure 1.6 The substrate cleavage specificities of MMPs

Enzyme	Substrate	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'
Collagenase	collagen (human) α1	G	P	Q	G	I	A	G	Q
	α2	G	P	Q	G	L	L	G	A
	collagenase (auto)	D	A	E	T	L	K	V	M
	collagenase (auto)	T	K	L	V	M	K	Q	P
	α_2-Macroglobulin (hum)	G	P	E	G	L	R	V	G
	PZP (hum)	Y	G	A	G	L	G	V	G
	α_2-Macroglobulin (rat)	M	D	A	F	L	E	S	S
	collagenase (hinge)	P	V	Q	P	I	G	P	Q
Stromelysin	stromelysin (auto)	D	T	L	E	V	M	R	K
	stromelysin (auto)	D	V	G	H	F	R	T	F
	collagenase	D	V	A	Q	F	V	L	T
	proteoglycan core	I	P	E	N	F	F	G	V
	α_1-proteinase inhibitor	E	A	I	P	M	S	I	P
	α_2-Macroglobulin (hum)	G	P	E	G	L	R	V	G
	substance P	K	P	Q	Q	F	F	G	L
Gelatinase	gelatin α1 fragment CB8	G	P	Q	G	V	R	G	E
	_	G	P	S	G	L	Hyp	G	P
	_	G	P	A	G	F	A	G	P
	gelatin α1 fragment CB7	G	P	Hyl	G	S	R	G	A
	gelatinase-A (auto)	D	V	A	N	Y	N	F	F
Matrilysin	insulin B-chain	L	V	E	A	L	Y	L	V
		E	A	L	Y	L	V	C	G
	matrilysin (auto)	D	V	A	E	Y	S	L	F
	matrilysin (auto)	R	V	I	E	I	M	Q	K
All MMPs	fluorescent peptide 1	McA	P	L	G	L	Dpa	A	R
	fluorescent peptide 2	Dnp	P	L	G	L	W	A	R

Fluorescent peptide 1 (Knight *et al.*, 1992)
Fluorescent peptide 2 (Stack and Gray., 1989)
Hyl = Hydroxylysine
Hyp = Hydroxyproline
PZP = Pregnancy zone protein

amino acids to the right of the cleavage site are designated P_1', P_2', P_3' etc. and interact with the corresponding S_1', S_2', S_3' sub-sites.

Collagenase invariably cleaves at the amino side of leucine or isoleucine. Whilst this cleavage often occurs after a glycine, other residues are not excluded (Fields *et al.,* 1987). For example, collagenase cleaves after a phenylalanine residue in the bait region of rat α-$_2$M (Sottrup-Jensen *et al.*, 1989) and following a proline (Birkedal-Hansen *et al.*, 1988) during the autolytic cleavage of the hinge region that may occur during activation or on prolonged storage .

Stromelysin tolerates a range of amino acid residues on either side of the cleavage site. Phenylalanine is the preferred P_1' target for its action on both itself and other members of the family. Harrison *et al.* (1989) have shown that stromelysin selectively cleaves a Q-F bond in the natural peptide, substance P, at a rate sufficient for its use as a substrate for screening assays. Very few amino acids including proline are excluded from the left of the cleavage site.

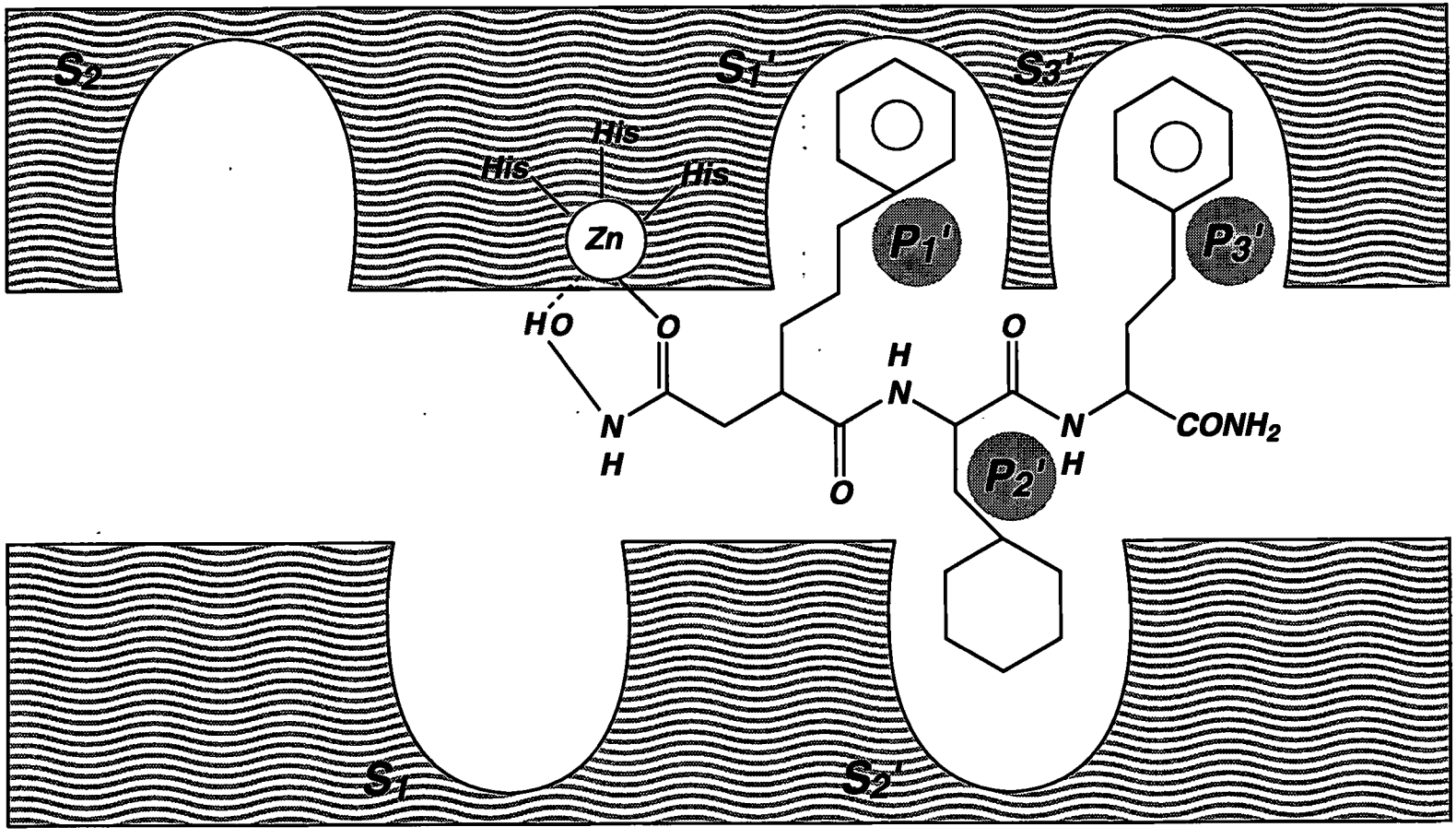
Like stromelysin, gelatinases cleave at a range of P_1' residues. Autocatalytic cleavages occur at tyrosine and phenylalanine suggesting a preference for large hydrophobic residues at this position. Gelatinases are more selective than stromelysin for residues to the left of the cleavage site. Small neutral amino acids are typically found at P_1 (Seltzer *et al.,* 1990) and gelatinase-A self-cleaves at an asparagine.

Matrilysin has a cleavage site specificity that is intermediate between that of collagenase and stromelysin. The activity of five MMPs including matrilysin was tested against a series of peptides by Netzel-Arnett *et al.* (1993). Their results indicate that whilst matrilysin can tolerate a wide range of amino acids at the P_1 position, it is particularly active against alanine containing peptides. Like collagenase, aliphatic amino acids such as leucine are preferred at the P_1' position. Surprisingly, during activation matrilysin can cleave itself at an E-Y bond in addition to the predicted cleavage of E-I and E-L (Crabbe *et al.*, 1992).

The fluorescent peptides developed by Stack and Gray, (1989) and Knight *et al.,* (1992) have a common G-L cleavage site for all the MMPs but the enzymes differ in their turnover rates of this substrate (Chapters 3 and 4).

Figure 1.7

A schematic representation of inhibitor-enzyme binding



The amino acid residues around a cleaved bond are named according to whether they are on the right-hand side (P_1', P_2', P_3') or the left-hand side (P_1, P_2, P_3) of that bond. These residues interact with the corresponding sub-sites (S_1, S_2, S_1', S_2', etc) on the enzyme. The same terminology is applied to inhibitors (above) which may also be classified according to whether they mimic the left-hand side (LHS) or right-hand side (RHS) of the cleaved substrate.

1.4: Inhibition of matrix metalloproteinases

1.4:1 General aspects of inhibitor design

The efficiency of a competitive peptidase inhibitor may be derived from the same combination of structural elements no matter which enzyme class is targeted. One element mimics the scissile bond and consequently blocks catalysis whilst others provide specificity by mimicking the structure of the substrate (Fig. 1.7). As a result of this convergence the majority of peptidase inhibitors look superficially similar. This means that a successful feature discovered in one area of drug development may be duplicated in the design of drugs from other unrelated areas. This is particularly evident in the structure of MMP inhibitors (MPIs) in which features of peptidyl-dipeptidase A (ACE), neprilysin, otherwise known as neutral endopeptidase (NEP) and renin inhibitors can readily be discerned.

In rational drug design the first step is to identify a compound that is active against the target enzyme. Early compounds may incorporate features derived from a knowledge of the substrate cleavage site, natural inhibitor structure or the cleavage mechanism. Having identified a lead compound it is then chemically modified to optimise its activity. By making small modifications of one feature at a time, for example, extension of a side-chain by one carbon, compounds can be assigned to homologous series. At the end of each series an optimised lead molecule can then be identified and this is continued until all the optimum characteristics have been incorporated. Having optimised the structure for potency the process is then repeated for selectivity, bioavailability and so on. In practice, the best structure for activity, for example, may conflict with that giving good bioavailability. This means that several different "structure-activity relationships" (SARs) may have to be constructed.

In addition to the approach outlined above the recent availability of crystallographic data for a number of MMP-inhibitor complexes provides additional information which may be used to rationalise further inhibitor modifications (section 1.4:4).

The concept of inhibiting metalloproteinases to give therapeutically useful compounds has originated in the ACE (Ondetti *et al.,* 1977) and neprilysin areas (Roques *et al.,* 1980). Many of the discoveries in these areas have been used to increase the potency of MMP inhibitors. Similar sources of information have

been used in the design of inhibitors with improved pharmacokinetic properties. Additionally, structural features used in the design of renin inhibitors have been incorporated in the design of pharmacokinetically superior MMP inhibitors. Since these areas have provided the basis for MMP inhibitor design a brief description of each will be presented.

Peptidyl-dipeptidase A (ACE) (EC 3.4.15.1)

ACE is a membrane-bound zinc metallopeptidase which catalyses the release of dipeptides from the carboxy-terminus of oligopeptide substrates. Its best known physiological function is the conversion of angiotensin I to angiotensin II by removal of the dipeptide H-L. It also catalyses the inactivation of the vasodilatory peptide bradykinin and cleaves Leu- and Met-enkephalins. Inhibitors are used in the control of hypertension.

Captopril (Fig.1.8) is an early example of drug design that was based on rational modifications to a chemical entity. Its design is derived from the structure of the active site of carboxypeptidase A obtained by X-ray crystallography and the structure of an ACE substrate, bradykinin (Ondetti *et al.,* 1977; Cushman *et al.,* 1977). Its success laid the foundation for the design of further ACE inhibitors such as enalaprilat (Fig. 1.8) as well as inhibitors of other metalloproteinases

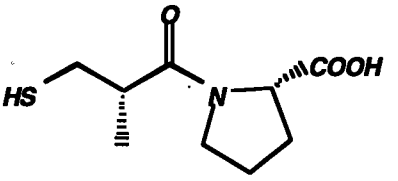
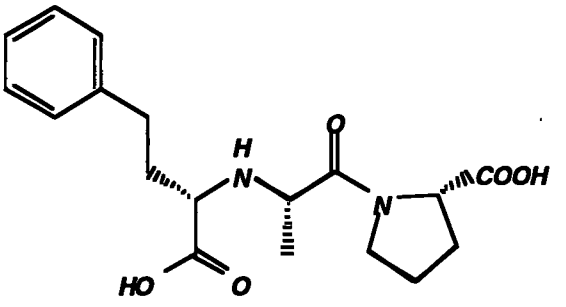
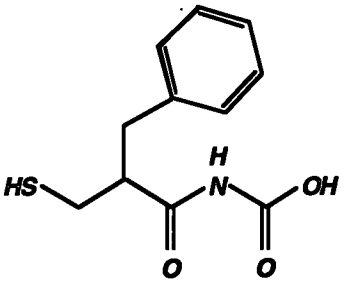
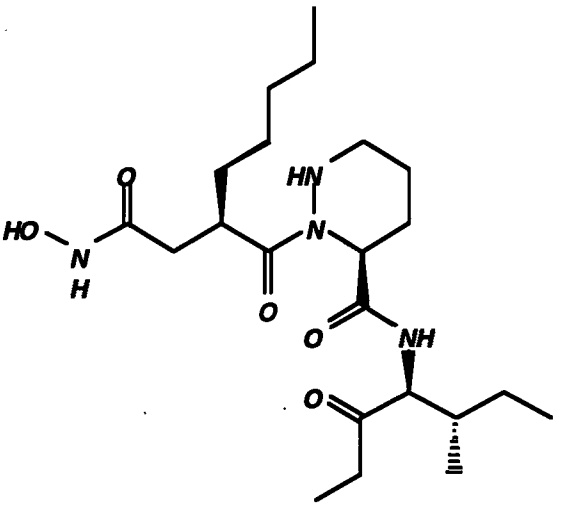
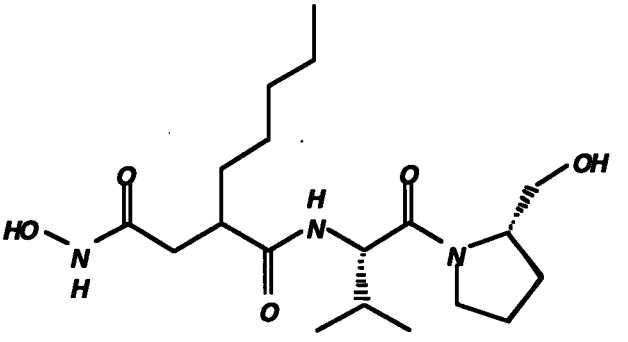
Neprilysin (NEP) (EC 3.4.24.11)

NEP is a membrane-bound zinc endopeptidase which also displays properties consistent with carboxypeptidase activity. It cleaves a broad range of substrates at the amino terminal side of hydrophobic residues such as phenylalanine, leucine and methionine. Inhibition of the enzyme has been shown to result in analgesia.

The first potent synthetic inhibitor of NEP was reported by Roques *et al.* (1980). This compound, thiorphan (Fig. 1.8) was based on captopril. Interestingly, NEP does not discriminate between [S]- and [R]- stereoisomers at the P_1' position and the P_1'-P_2' peptide bond can even be reversed (retrothiorphan) without a reduction in activity. This distinguishes NEP inhibitors from those of ACE and the MMPs (see Chapter 5). Carboxylic acid inhibitors of NEP were described by Mumford *et al.* (1982). The esterification of the carboxylic acid group to generate a prodrug has been explored by Roques *et al.* (1993) as a means of modifying the distribution of these inhibitors. This approach

Figure 1.8

Metalloproteinase inhibitors

Compound	Structure	Source
Captopril		Synthetic
Enalaprilat		Synthetic
Thiorphan		Synthetic
Matlystatin B		Actinomycetes
Actinonin		Actinomycetes

had previously been used successfully in the ACE area with enalapril, which is the ester prodrug of the carboxylic acid inhibitor, enalaprilat. The introduction of a hydroxamic acid zinc binding ligand separated from the P_1' group by a methylene spacer (Bouboutou *et al.*, 1984), provided a structural format for the design of MMP inhibitors which is still in use.

Renin

Renin is an aspartyl proteinase which catalyses the production of angiotensin from angiotensinogen. Interest in the enzyme stems largely from the success of ACE inhibitors which block the action of ACE at a later stage in the renin-angiotensin pathway. The poor *in vivo* characteristics encountered with these peptide based drugs has been a major obstacle to the clinical use of renin inhibitors. Workers in this area have, therefore, been particularly active in their attempts to overcome these problems. The recognition of a need to balance the hydrophilic and hydrophobic elements to achieve oral bioavailability has resulted in the development of a large number of non-amino acid side-chains. These side-chains which include morpholine amides, sulphamylurea and arylsulphonamides (Patt *et al.*, 1992; Ashton *et al.*, 1992; Doherty *et al.*, 1992) have now been incorporated into a range of MMP inhibitors by several industrial groups.

1.4:2 Natural protein inhibitors of MMPs

The most important inhibitors of MMPs *in vivo* are the ubiquitous α-$_2$ macroglobulin and a family of specific inhibitors, TIMPs 1, 2 and 3 (Stricklin and Welgus, 1983; De Clerck *et al.*, 1989; Uria *et al.*, 1994). The former traps enzymes using a proteinase sensitive bait sequence (Barrett and Starkey, 1973). Following cleavage of this sequence the inhibitor collapses around the enzyme and prevents its access to large substrates.

TIMPs range in size from 20 - 30 kDa but this is largely a result of glycosylation. They are highly potent reversible inhibitors of MMPs with inhibition constants (Ki's) in the picomolar range (Murphy *et al.*, 1992a). The structure of TIMP is maintained by six disulphide bonds. Two distinct domains can be distinguished by their relative abilities to bind to active gelatinase and to progelatinase (Murphy *et al.*, 1991). The N-terminal domain appears to carry the minimum information required for inhibitory activity whereas the C-terminal domain is required for binding of TIMP-1 to progelatinase-B and TIMP-2 to

progelatinase-A. Reduction of the disulphide bonds leads to the complete loss of TIMPs ability to inhibit stromelysin in a rat pleural cavity model (Lark *et al.*, 1990) suggesting multiple interactions between the inhibitor and enzyme. This places severe limitations on TIMP's application to the design of low molecular mass inhibitors since its potency derives from the contribution of interactions from distant sites of the primary sequence as opposed to a single bait region. Nonetheless, TIMPs have been valuable tools *in vitro* to demonstrate a role for MMPs in tumour and endothelial cell invasion of tissue matrices (DeClerck *et al.*, 1991; Mignatti *et al.*, 1989), angiogenesis (Moses and Langer, 1991) and *in vivo* models of cartilage destruction and metastasis (Carmichael *et al.*, 1989; Alvarez *et al.*, 1990).

1.4:3 Inhibitors from naturai sources

Several inhibitors of MMPs have been isolated from the screening of microbial broths. Actinomycetes have been a particularly rich source of such inhibitors producing the tetracyclines described by Golub *et al.*, 1984 in addition to a series of hydroxamic based inhibitors including the matlystatins and actinonin.

The matlystatins are a family of low molecular mass MMP inhibitors isolated from a strain of actinomycetes by Ogita *et al.* (1992). The structure of matlystatin B is presented in Fig. 1.8. These compounds are active in the low micromolar range against a variety of zinc metalloproteinases including thermolysin, aminopeptidase M, interstitial collagenase, gelatinase-A and stromelysin for which they are particularly potent (IC_{50} = 80 nM). Matlystatin A inhibits the invasion of the artificial basement membrane, matrigel, by HT 1080 human fibrosarcoma cells with an IC_{50} value of 21.6 μM.

Actinonin (Fig. 1.8) is a pseudo-peptide antibiotic. It was identified from the anti-collagenase activity (Ki = 1.4 μM) of actinomycetes by workers at Rhone-Poulenc (Faucher *et al.*, 1987). However, actinonin is now known to be a more potent inhibitor of the zinc aminopeptidases, particularly aminopeptidase N (AP-N; CD13).

There are remarkable structural similarities between the matlystatins, actinonin and synthetic inhibitors of MMPs. The former are generally less peptidic in nature and so may provide some information to aid the design of non-peptide analogues of MMP inhibitors.

1.4:4 The design of MMP inhibitors

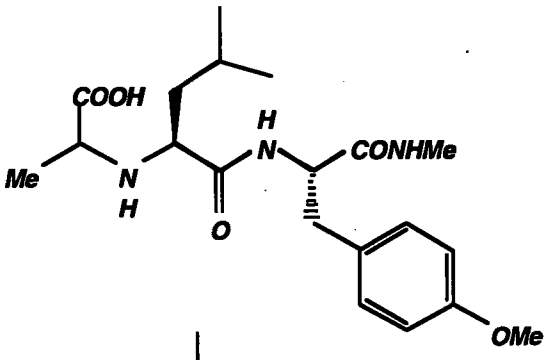
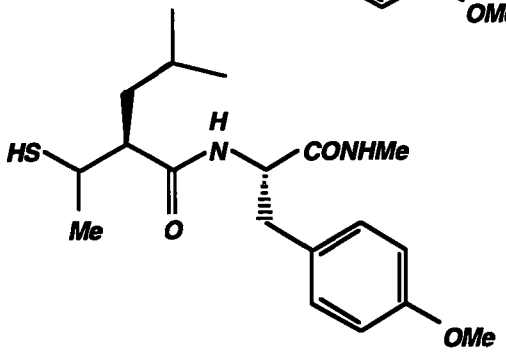
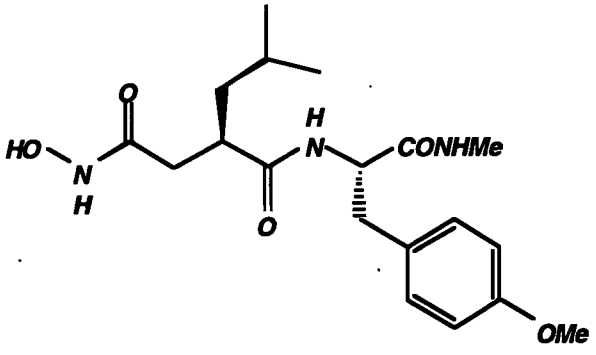
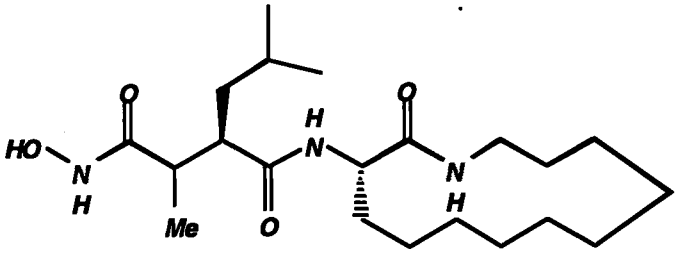
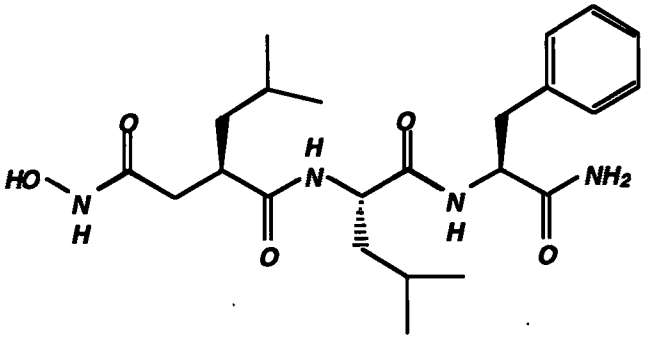
(i) Experimental approaches to inhibitor design

In the early 1980's a number of pharmaceutical companies began to develop inhibitors of interstitial collagenase. The rationale for this choice of enzyme target was based on the unique ability of collagenase to degrade the collagen of bone and cartilage which implied an important role in diseases of matrix destruction such as arthritis.

The first group to report the synthesis of rationally designed collagenase inhibitors was that of Gray *et al.* (1981). By direct analogy with ACE inhibitors a sulphydryl group was attached to a C-terminally blocked peptide which was loosely based on the residues to the right-hand side of the collagen cleavage site. Early efforts focused on the design of inhibitors based on both the left-hand side (LHS) and the right-hand side (RHS) of the cleavage site (reviewed in Johnson *et al.,* 1987). These studies demonstrated that modifications to RHS inhibitors could produce improved potency. The results of the G.D. Searle group are typical of the developments made in the MMP area over the following years. The inhibitor side-chains were optimised for collagenase inhibition by incorporating a tyrosine derivative at the P_2' position and reducing the size of the P_3' to methylamide. It became evident, however, that the key to potency lay in the choice of the zinc-binding group. Early inhibitors included a substituted N-carboxymethyl for this purpose (McCullagh *et al.,* 1984). The most potent of these (compound 1) had an IC_{50} of 0.8 μM (Fig. 1.9). During this period improvements in chiral separation lead to the important discovery that collagenase preferred R stereochemistry at the P_1' position and this discriminated it from ACE. Replacement of N-carboxylalkyls with thiol zinc ligands, produced inhibitors (compound 2) with IC_{50} values of 0.2 - 0.3 μM (Donald *et al.,* 1985). No further progress was made until the introduction of hydroxamic acid ligands which had been used successfully in the NEP area (Fournie-Zaluski., 1985). Introduction of this zinc ligand onto the optimised Searle peptide back-bone (compound 3) produced an inhibitor with an IC_{50} of 0.02 μM (Dickens *et al.,* 1986). Investigators at Roche achieved similar

Figure 1.9

The design of collagenase inhibitors

Compound	Structure	IC50 [μM]
1 (Searle)		0.8 (collagenase)
2 (Searle)		0.2 (collagenase)
3 (Searle)		0.02 (collagenase)
4 (Roche)		0.026 (collagenase)
5 (ICI)		0.4 (collagenase) 0.04 (stromelysin)

potencies (0.03 μM) with a series of phosphinic acid based compounds (Borkakoti *et al.*, 1988). Inhibitors based on a model of collagen provided evidence that the P_2' and P_3' residues could be cyclised in a trans-amide conformation without loss in potency. At the same time, selectivity for collagenase was provided by the presence of a large cyclic imido group in the P_1 position. Cyclisation of the P_2' and P_3' residues was a significant move away from the traditional dipeptide arrangement and its incorporation into hydroxamic acid based inhibitors produced a non-peptide compound (compound 4) with similar potency to the parent compound.

Hydroxamic acid inhibitors represented the state of the art in 1986 and indeed since then, there have been few changes to the basic structures of these compounds. Many of these early studies, however, were carried out on collagenase of questionable purity, isolated in small amounts from natural sources such as pig and rabbit. Consequently, the cloning of each of the MMP cDNAs leading to a full panel of pure human recombinant enzymes including collagenase (Goldberg *et al.*, 1986), stromelysin (Whitham *et al.*, 1986), gelatinase-A (Collier *et al.*, 1988), gelatinase-B (Wilhelm *et al.*,1989) and matrilysin (Busiek *et al.*,1992) was a significant step forward. One of the earliest examples of an inhibitor that had been screened for selectivity was the ICI analogue (compound 5) which showed some preference for stromelysin (Di Pasquale *et al.*, 1986). Although inhibitors could now be evaluated against a number of individual enzymes the full potential of this technology was not realised until the development of improved peptide screens. The fluorescent peptide substrates developed by Stack and Gray, (1989) and Knight *et al.* (1992) formed the basis for fully automated screens which were robust enough to permit Ki's to be established. This was particularly important because potencies were now in the low nanomolar range. Such potent inhibitors titrate the enzyme at concentrations which are required to hydrolyse collagen and the resulting IC_{50} value becomes equivalent to 50% of the enzyme concentration (see Chapter 6).

These developments have been exploited at Celltech to produce a selection of potent inhibitors with a range of selectivities. It was recognised that the cleavage specificity of collagenase was not representative of the other members of the family. Inspection of the substrates led to the development of inhibitors with an aryl group at the P_1' position (Porter *et al.*, 1992). This conferred gelatinase and stromelysin selectivity over interstitial collagenase together with

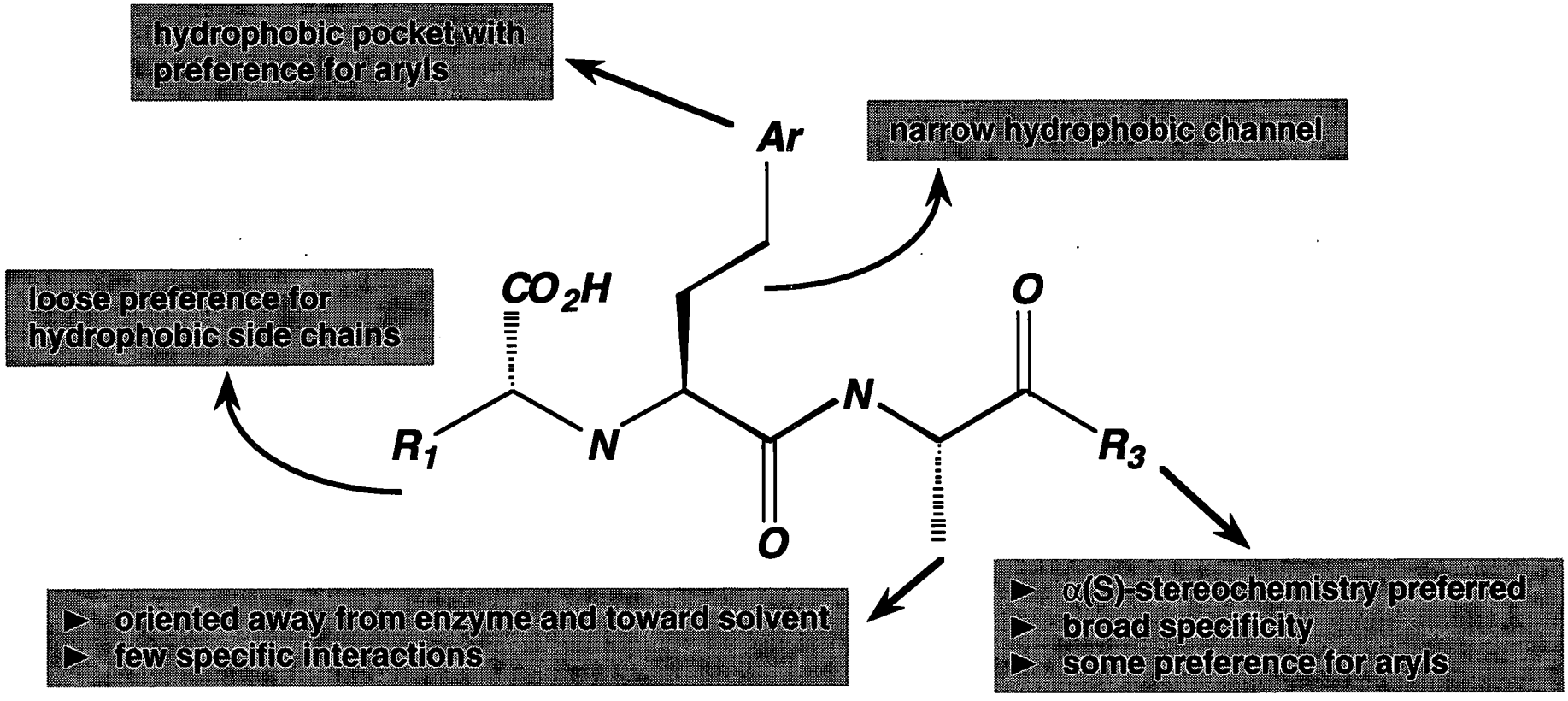
significant improvements in potency (Chapter 5). The studies reported by Chapman *et al.* (1993) confirm these observations. They constructed SARs for a series of N-carboxyalkyl inhibitors against stromelysin, collagenase and gelatinase-A. The importance of interactions at the S_1' sub-site was revealed by the introduction of a phenethyl group at the P_1' position. This produced a compound with an affinity against stromelysin (Ki = 6.5 nM) which is normally associated with inhibitors possessing a hydroxamic acid zinc ligand. Figure 1.10 summarises the conclusions of these SAR studies.

(ii) MMP inhibitor design from 3-dimensional structures

As described earlier the structures of several MMPs have now been solved using X-ray crystallography and NMR spectroscopy. The inclusion of inhibitors in some of the studies permits enzyme-inhibitor contacts to be identified and the amino acid composition of the enzyme sub-sites to be assigned. Of particular relevance to this study are the descriptions of RHS inhibitor binding. Figure 1.11 (A) shows the hydrogen bond contacts of a synthetic hydroxamic acid inhibitor co-crystallised with the catalytic domain of interstitial collagenase (Borkakoti *et al.*, 1994). Two oxygens from the hydroxamic acid bind to the zinc so that the metal ion is penta-co-ordinated (trigonal-bipyramid). Additional hydrogen bonding provided by the NH- group to a backbone carbonyl explains the superior potency of the hydroxamate ligand over other bi-dentate ligands. All the backbone groups are hydrogen bonded which is consistent with the experimental observation that inversion of peptide bonds is not tolerated by these enzymes. The conformation of the bound synthetic inhibitor has the P_2' side-chain directed away from the enzyme and on the opposite side of the inhibitor backbone to the P_1' side-chain. This confirms the validity of the modelling studies used by Roche who arrived at the same conclusion using a repeating tri-peptide template based on collagen (reviewed in Johnson *et al.*, 1987) In order to probe the nature of the substrate binding pockets Stams *et al.* (1994) compared the crystal structure of the catalytic domain of interstitial collagenase with its neutrophil counterpart. The most notable difference was the larger size of the S_1' sub-site of the neutrophil form. This appears to be a consequence of the replacement of an arginine (R^{195}) with a leucine. In the interstitial collagenase R^{195} points towards the catalytic zinc, effectively closing the end of the S_1' pocket. In the neutrophil enzyme the corresponding residue, leucine, is orientated away from the zinc

Figure 1.10

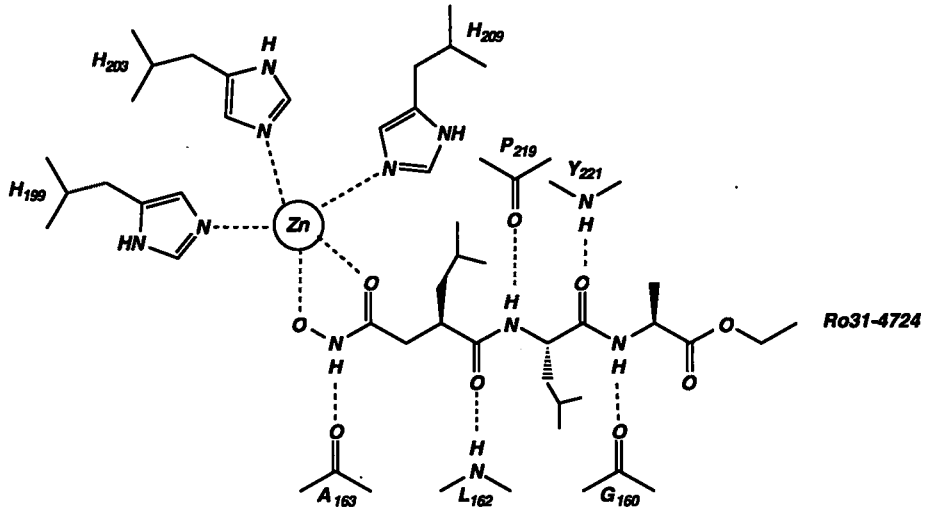
Summary of the structure-activity relationship for inhibition of matrix metalloproteinases by N-carboxyalkyl peptides



Taken from Chapman et al (1993)

Figure 1.11 MMP - inhibitor interactions

A



B

ENZYME	162			A					170	196			B					205	219		C				225
Stromelysin	N	V	L	A	H	A	V	A	P	F	L	V	A	A	H	E	I	G	H	M	Y	P	L	Y	H	S
HFC	G	N	L	A	H	A	F	Q	P	H	R	V	A	A	H	E	L	G	H	M	Y	P	S	Y	T	F
GL-B	G	L	L	A	H	A	F	P	P	F	L	V	A	A	H	E	F	G	H	M	Y	P	M	Y	R	F
GL-A	G	L	L	A	H	A	F	A	P	F	L	V	A	A	H	E	F	G	H	M	A	P	I	Y	T	Y
HNC	G	I	L	A	H	A	F	Q	P	F	L	V	A	A	H	E	F	G	H	M	Y	P	N	Y	A	F
Matrilysin	N	T	L	A	H	A	F	A	P	L	Y	A	A	T	H	E	L	G	H	M	Y	P	T	Y	G	N
	61								69	87								96	147						153
Astacin	G	S	G	C	W	S	Y	V	G	H	G	T	I	I	H	E	L	M	H	M	H	Y	G	K	Y	S
	106								114	137								146	166						172
Adamalysin	K	T	L	G	K	A	Y	L	N	A	V	T	M	A	H	E	L	G	H	M	R	P	G	L	T	P
		S_2'	S_3'								S_1'	S_1'										S_1'	S_3'	S_1'		

Zinc box

(A) Schematic representation of the hydrogen bond interactions (dashed lines) between the inhibitor RO 31-4724 and the catalytic domain of fibroblast collagenase. Interactions with the catalytic zinc are also shown. (from Borkakoti et al., 1994)

(B) Alignment of portions of the amino-acid sequences of six human MMPs, fibroblast collagenase (HFC), stromelysin-1, gelatinase-G (GL-B), gelatinase-A (GL-A), neutrophil collagenase (HNC) and matrilysin together with, astacin and adamalysin II. The alignment is based on the sequence and structural homology of the catalytic domain of stromelysin where (A) is from strand IV, B, the HEXXH of helix B and C, the Met turn. Amino-acid residues forming sub-sites S_1' - S_3' are indicated. (from Gooley et al., 1994)

resulting in an enlarged pocket. This is consistent with the observation that neutrophil collagenase is active on substrates with aromatic groups in this position (Hasty *et al.,* 1987). These studies were complemented by the work of Gooley *et al.* (1994) who used NMR to determine the 3-D structure of a truncated stromelysin complexed with an N-carboxyalkyl inhibitor. Amino-acid residues involved in substrate/inhibitor recognition were identified and this was extended to other metzincins using sequence homologies (Fig.1.11{B}). Amongst the MMPs only interstitial collagenase and matrilysin possess a residue other than leucine at position 197 (corresponding to R^{195} in interstitial collagenase). From the description given above we can see that this is consistent with the preference of other members of the family for an aromatic residue at the P_1' position and stresses the importance of this residue in delineating the S_1' pocket. Only one residue (V^{163} in stromelysin) can be assigned to the S_2' sub-site suggesting that this is a very shallow pocket. Two residues are assigned to the S_3' sub-site, one of which varies significantly between MMP members. This indicates that specificity may be built into the P_3' position although the openness of the pocket may make the enzymes tolerant to a range of substitutions. These conclusions are entirely consistent with the SARs reported by Chapman *et al.* (1993) and the studies conducted at Celltech (Porter *et al.,* 1992).

1.4:5 The kinetics of MMP inhibition

The potency of MMP inhibitors are typically described in terms of either their IC_{50} value or their inhibition constant (Ki). The former refers to the concentration of inhibitor which reduces enzymic activity by fifty percent. This form of measurement is commonly employed during the establishment of a study. At this stage, inhibitors may be relatively inefficient and the measurement of relative inhibitory activity may be of more importance than absolute potency. The expression is, however, relatively uninformative since it depends on the type of inhibition and the experimental conditions under which it is determined. The continuing use of the term in the MMP area derives from the use of collagen in assays. This is because collagen exhibits varying degrees of solubility making it unsuitable for kinetic studies which require an accurate measurement of substrate concentration.

The Ki is a more exact term and defines the dissociation of the inhibitor-enzyme complex or the reciprocal of the binding affinity to the enzyme.

The greater control over the assay conditions afforded by peptide substrates permits the kinetics of enzyme-substrate-inhibitor interactions to be studied in great detail. In doing so it is likely that investigators assume that the enzyme-inhibitor interaction is reversible and indeed the use of the term Ki, presumes this to be the case. The absence of any inhibitor group with obvious potential for covalent interactions with the enzyme suggests that this may be an accurate assumption and indeed the reversibility of stromelysin inhibitors has now been demonstrated experimentally (Izquierdo-Martin and Stein, 1992).

Morrison (1982) defined four main classes of inhibitor on the basis of reversibility, potency and the rate of inhibitor-enzyme reaction, namely; classical, tight-binding, slow-binding and slow, tight-binding.
The Ki value for a classical, competitive inhibitor may be determined using the Michaelis-Menten relationship;

$$v_i = \frac{V_{max}.S}{K_m (1 + [I]/Ki) + S}$$

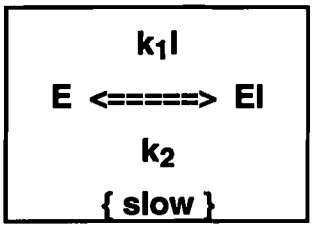
where v_i is the velocity in the presence of inhibitor, V_{max} is the maximum velocity, K_m is the Michaelis constant for the substrate, S, and [I] is the concentration of inhibitor.

Tight-binding inhibition is characterised by inhibition at inhibitor concentrations comparable to those of the enzyme. This means that data cannot be analysed using the equation for classical inhibition since this assumes that the free inhibitor concentration equates to the total inhibitor concentration. As the majority of MMP inhibitors have nanomolar Ki's they presumably fall into this category. Accordingly, the treatment of kinetic data must allow for the mutual depletion of enzyme and inhibitor.. A general steady-state rate equation that accounts for the tight-binding of inhibitor to enzyme was derived by Morrison (1969) (reviewed in Williams and Morrison, 1979 and Morrison and Walsh, 1988) (see section 2.2:4).

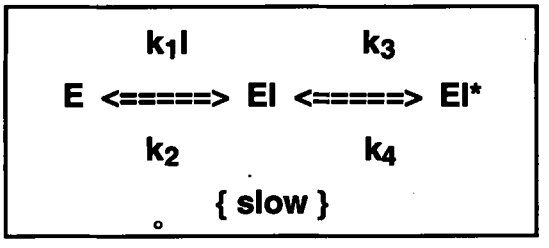
An inhibitor may be considered slow-binding if its potency increases on a time-scale (i.e. longer than seconds) that is readily followed by conventional assay (Schloss, 1988). Slow, tight-binding inhibition is characterised by a time-dependent inhibition of the enzyme at inhibitor concentrations comparable to those of the enzyme. Most early references to this phenomenon, reported association rates (reviewed in Schloss, 1988) that were considerably lower than

the diffusion controlled limit of 10^7 - 10^9 $M^{-1}s^{-1}$ (Brouwer and Kirsch, 1982; Hardy and Kirsch, 1984). The first kinetic treatment of slow, tight-binding inhibition was provided by Cha (1975) (also Cha *et al.*, 1976) who proposed two mechanisms to account for the slow establishment of equilibrium assuming no change in inhibitor form.

In mechanism A, enzyme and inhibitor bind in a single slow step which is defined by an association rate constant (k_{on}) and a dissociation rate constant (k_{off}). A progress curve of product formation with time in the presence of enzyme and inhibitor can be described by a single steady-state velocity (v_s). The substrate concentration may be disregarded at concentrations significantly lower than the K_m and so this process may be described as follows,



In mechanism B, inhibitor [I] binds rapidly to the active site of the enzyme to produce an EI collision complex. This now undergoes a relatively slow isomerism to produce a modified complex, EI* (tightened complex).



These two mechanisms exhibit different characteristics which are discussed in Chapter 6.

A number of studies have shown that slow, tight-binding may be a common feature of inhibitors with very low Ki's (Morrison and Walsh, 1988). The binding of the folate analogue, methotrexate, to dihydrofolate reductase (Williams *et al.*, 1979) is a prominent example of such a mechanism. Perhaps, the most

extensively documented examples in the metalloproteinase field are the phosphorus based inhibitors of thermolysin (Bartlett and Marlow, 1987). The association rates of these inhibitors are of the order of 10^3 $M^{-1}s^{-1}$. Similar compounds have also been shown to bind slowly (k_{on} = 10^4 $M^{-1}s^{-1}$) to stromelysin (Izquierdo-Martin and Stein, 1992). The slow binding of these so-called 'transition-state analogues' is presumed to be a consequence of the slow exchange of inhibitor for zinc-bound water. This may not, however, represent the mechanism by which simple 'ground-state' substrate analogues bind.

In a study conducted by Izquierdo-Martin and Stein (1994), N-carboxyalkyl inhibitors were shown to bind slowly to stromelysin with k_{on}'s of the order of 10^4 $M^{-1}s^{-1}$ but this series may, like the phosphorus based inhibitors, be classed as transition state analogues. There are no reports of the kinetics of hydroxamate inhibitor binding to MMPs.

There are several descriptions of slow, tight-binding inhibitors in the ACE area. Bull *et al.* (1985) compared the slow binding of captopril (thiol), and enalaprilat (carboxylate) and lisinopril (carboxylate) to ACE. Whilst these compounds exhibit time-dependent inhibition the association rate constants (k_{on} = 10^6 $M^{-1}s^{-1}$) are arguably within diffusion controlled limits. Similarly, idapril (hydroxamic acid) has also been described as slow binding despite a k_{on} of 3 x 10^6 $M^{-1}s^{-1}$ (Lippi *et al.*, 1993). These compounds are highly active and this illustrates another aspect of slow-binding kinetics. For inhibitors with Ki's of the order of 1 nM the slow onset of equilibrium can be a consequence of a slow rate of dissociation even if the association rate is diffusion limited. Bartlett and Marlowe (1987), therefore, suggest an upper limit of 10^6 $M^{-1}s^{-1}$ for a true slow-binding mechanism. Experimentally, tight-binding inhibitors may be studied using slow-binding kinetics by decreasing the enzyme concentration (which is dependent on having a high substrate turnover number). This is an important method for determining the values for k_{on} and k_{off} of such inhibitors (Morrison, 1982). The application of these techniques to MMP inhibitors will be described in Chapter 6.

1.4:6 The pharmacokinetics of MMP inhibitors

The efficacy of an orally delivered drug is related to its efficiency in traversing the intestinal membrane (absorption), its pre-systemic elimination and its systemic half-life. These factors have presented a major challenge to the

clinical development of peptidic drugs (Humphrey and Ringrose, 1986). Oral bioavailability (often expressed as a percentage) is typically defined in terms of the ratio of orally administered drug detected in the plasma to that detected after administration by the intravenous route.

The design of inhibitors of the renin-angiotensin system have served as models for the design of orally active peptidic drugs in general. Three main approaches have been employed, namely;

(1) The use of prodrugs that are converted to the active species *in vivo.*

(2) Depeptidisation

(3) Modifications of the lipophilic/hydrophilic balance

A combination of the first two approaches has proved successful in the development of enalaprilat (Figure 1.8). The pharmacokinetic properties of this compound were significantly improved by blocking the zinc-binding carboxylate group to form the prodrug, enalapril. After absorption from the gastrointestinal tract the blocking group is readily removed by esterase action.

Depeptidisation has long been a goal of workers in the MMP area. Cyclisation of the P_2' and P_3' groups without a loss in potency was achieved by Roche (section 1.4:4), however, complete depeptidisation has not been achieved.

Passive absorption across the gastrointestinal tract requires a correct balance of lipophilic and hydrophilic elements. Modification of the physiochemical properties of compounds to achieve this balance has been extensively studied in the renin area. Boyd *et al.* (1992) reported the use of a wide variety of basic groups to produce a series of soluble and bioavailable renin inhibitors which retained their potency. Other groups achieved oral bioavailability by incorporating histidine-like substituents into their structures (Patt *et al.,* 1992)

Kleinert (1992), however, showed that peptide-like inhibitors of renin of differing sizes were efficiently absorbed in several animal species (reviewed in Kleinert *et al.,* 1993). They concluded that the major cause of poor bioavailability in such drugs was rapid hepatic clearance.

The problems associated with peptide based drugs is particularly evident in the MMP area where a lack of oral bioavailability and poor systemic half-life have severely hindered progress into the clinic. These obstacles are reflected in the notable absence of pharmacokinetic literature on the subject.

Studies of the Searle collagenase hydroxamic inhibitor (Compound 3, Fig. 1.9) demonstrated its lack of oral bioavailability in the rat despite its stability over 24 hours in the blood (Singh *et al.,* 1993). Perfusion using organ baths demonstrated that the compound was adequately absorbed across the rat intestine but neither the parent nor its major metabolite, the carboxylic acid, escaped the liver. The C-terminal region of the drug was then targeted as a means of altering its physiochemical properties without affecting potency. The result was a series of compounds with oral bioavailabilities ranging from 0 - 10 % and systemic half-lives ranging from 6.9 - 43.7 minutes. A more detailed study of the metabolism of a related drug BB94 (batimastat) was undertaken by Yachetti *et al.* (1993). Several metabolites were identified in the bile shortly after intravenous administration. The major site of biotransformation was shown to be the hydroxamic acid.

Some progress was made by the incorporation of a tertiary butyl group into the P_2' position of a collagenase inhibitor (Ro31-9790) developed by Roche (Meeting report by Lewis *et al.,* 1993). The oral bioavailabilities of this inhibitor were estimated to be 7% and 17% in the rat and marmoset, respectively. The compound which is a broad spectrum collagenase and gelatinase inhibitor is also reported to possess a systemic half-life of about 1 hour in the rat.

Despite its use in clinical trials for the treatment of ascites the British Biotech compound, batimastat, is not reported to be orally bioavailable and is currently administered by the intraperitoneal (i.p.) route. A series of modifications to the compound have been patented (Dickens *et al.*, 1994) with the claim that replacing the α-carbon substituent (see Fig. 5.1) with a hydroxyl group leads to improvements in oral bioavailability. The pharmacokinetics of gelatinase-A inhibitors is the subject of Chapter 7.

1.5: Experimental approach

The contents of this thesis can be divided into two sections. The first part, Chapters 3 and 4, deal largely with the activation of progelatinase-A, progelatinase-B and interstitial procollagenase. The second part (Chapters 5, 6 and 7) covers the processes by which inhibitors to these MMPs were designed.

Methods for activating MMPs are widely reported in the literature (reviewed in Birkedal-Hansen *et al.*, 1993) and indeed activation by APMA is a

characteristic property of this family. Nevertheless, the activation of progelatinase-A, progelatinase-B and procollagenase (enzymes provided by Dr. T. Crabbe) has been the subject of extensive debate. Accordingly, chapters 3 and 4 address the following issues;

(1) The resistance of progelatinase-A to test-tube activation by proteinases.

(2) The contribution of the C-terminal domain to progelatinase-B activation

(3) The phenomenon of the "superactivation" of collagenase

A combination of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorescent peptide assay were used to study the activation process. This allows the enzymic activity to be related to the enzyme form, since activation is generally associated with a reduction in molecular mass. In many cases the identities of enzyme species were also determined by N-terminal sequencing. This work showed that progelatinase-A may be activated in the test-tube by either matrilysin or collagenase. The latter is not able to complete the process and, by using a specific inhibitor of gelatinase-A, its cleavage site was identified. It proved to be identical to an intermediate arising from the activation of progelatinase-A by stimulated human fibrosarcoma cells (Strongin *et al.*, 1993). The action of collagenase was then shown to be reciprocated because collagenase became superactivated by co-incubation with progelatinase-A. The whole process was stimulated by heparin, which may act as a template for the two enzymes by binding to their C-terminal domains.

Superactivation, correlates with the appearance of F^{81} at the N-terminus of collagenase. It was first noted by Murphy *et al.* (1987) when interstitial collagenase was incubated with stromelysin. Work in this thesis demonstrates that this phenomenon is limited to increasing the rate at which collagenase cleaves collagen and has no effect on the peptidase activity of collagenase. This is relevant to the role of F^{81} in promoting superactivation, which will be discussed in Chapters 4 and 8 in the light of recent crystallographic studies.

The contribution of the C-terminal domains to activation and activity was examined using genetically engineered proteins that lacked this domain. The results show that truncated enzymes demonstrate identical activities to the parent enzymes apart from the inability of truncated collagenase to cleave collagen. This was expected since similar results have been described by other groups (Birkedal-Hansen *et al.*, 1988; Clark and Cawston, 1989). The observation was

taken a step further, however, by demonstrating that a monoclonal antibody (MAC065), directed against the C-terminal domain of collagenase, specifically blocked collagenolysis. Collagen binding may, therefore, provide an additional target for the design of collagenase inhibitors.

Fluorescent peptide assays were developed for gelatinase-A, gelatinase-B, collagenase, stromelysin and matrilysin. Appropriate assays were also established for two zinc peptidases, ACE and AP-N. Chapter 5 shows how these assays can be used to construct a series of inhibitor SARs, which are important to the rational design of new chemical entities. Optimisation of inhibitor side-chains led the Celltech Chemistry Department to synthesise compounds with Ki's of less than 10^{-11} M against the target enzyme, gelatinase-A, and which are of the order of 50,000 fold more potent against this enzyme than interstitial collagenase. These experimentally derived SARs may be further rationalised using the enzyme structure data that has since become available (see section 1.4:4 {ii}).

A kinetic treatment of the binding of phosphorus-based inhibitors and N-carboxyalkyl inhibitors to stromelysin has been reported by Izquierdo-Martin and Stein, (1992; 1994). These inhibitors exhibit association rate constants (k_{on}) of the order of 10^4 $M^{-1}s^{-1}$, which is a characteristic of slow-binding kinetics. There are, however, no reports on the binding of hydroxamic acid inhibitors to MMPs, although the ACE literature does contain examples of such compounds (Lippi *et al.*, 1993). Complex formation between MMP inhibitors and gelatinase-A was analysed under pre-steady-state conditions (Chapter 6). The time-dependency of inhibition, which arises from the use of low reactant concentrations, allowed the data to be analysed using slow-binding equations. The k_{on} for a number of inhibitors was in the range of 10^6 - 10^7 $M^{-1}s^{-1}$ which is consistent with diffusion limited association. These values are comparable to that reported for the ACE hydroxamic acid inhibitor, Idapril (Lippi *et al.*, 1993). The dissociation half-lives of all the compounds tested were of the order of minutes to hours so the high affinity of hydroxamic acid inhibitors for gelatinase-A is the product of a slow rate of dissociation and a fast rate of association.

There is little published data concerning the pharmacokinetics of MMP inhibitors. The data that is available (section 1.4:6) suggest that the systemic half-lives and oral bioavailability of hydroxamic inhibitors are poor in the rat. The same principles apply to the design of pharmacokinetically active drugs as for the design of good *in vitro* properties so in order to construct SARs, a high-

throughput screen is necessary. This was provided by a mouse pleural cavity assay (section 2.4:3), which was developed in collaboration with the Dr. P. Antoniw, Dr. S. Chander and Dr. N. Willmott of the Celltech Oncology Pharmacology Department. This assay identified a series of systemically active and orally active inhibitors but its narrow concentration-effect range meant that it was not quantitative (Chapter 7). Phamacokinetic properties were, therefore, determined by the standard practice of measuring inhibitor levels in the blood. The lack of a distinguishable chromophore, ruled out the direct measurement of inhibitor concentration. Instead, the inhibitor was extracted from the blood and its inhibitory activity measured either by titration against gelatinase-A (section 2.4:2 {i}) or by Capillary Electrophoresis (2.4:2 {ii}). These studies resulted in the identification of inhibitors with oral bioavailabilities of about 40% in the mouse and 7% in the rat.

Chapter 2

CHAPTER 2

MATERIALS AND METHODS

2.1: General materials

2.1:1 Reagents, stock solutions and equipment

General chemicals

General chemicals were purchased from B.D.H Ltd., Poole, Dorset, or Sigma Chemical Co., Poole, Dorset, unless stated otherwise. Methanol, acetonitrile and HPLC grade water were obtained from Fisons, Loughborough. Radioisotopes were purchased from Amersham International, Little Chalfont, Bucks.

APMA (Sigma A-8514) Stock solution of 20 mM in DMSO. Stored at room temperature.

Bacterial collagenase (Sigma C-9891) type 1A from *Clostridium histolyticum*. Stock solution is 10 mg/ml in water. Stored at -20°C.

Brij 35 (Pierce 28316) 10% aqueous solution stored at room temperature.

Brij 35 (Sigma Diagnostics 430AG-6) 30% solution (w/v) stored at room temperature.

Heparin (Sigma H-3125) Sodium salt, grade I from porcine intestinal mucosa. Stored as powder at room temperature.

p-Dioxane Dried over Molecular sieve 4A (stored in fume cupboard)

1,10-Phenanthroline solution 16 mg of 1,10-phenanthroline monohydrate dissolved in 0.5 ml p-dioxane and diluted to 1 ml with deionised water. (Prepared fresh for each experiment)

PMSF (Sigma P-7626) Stock solution of 100 mM in propan-2-ol stored at room temperature.

SBTI (Boehringer Mannheim 109 886) Stock solution is 4.5 mg/ml in peptide assay buffer. Stored in 50 µl aliquots at-20°C

Scintillation fluid Aqua luma plus (Lumac LSC)

TCA Stock solution is 90% (w/v) in water.

Trypsin (Sigma T-8642) Type XIII :TPCK-treated trypsin from bovine pancreas. Stock solution of 0.1 mg/ml in 10 mM HCl, 10 mM $CaCl_2$. Stored in 0.1 ml aliquots at -20°C.

Immobilon membrane for western blotting (Millipore) Membranes are presoaked in methanol for a few seconds and then washed in transfer buffer prior to use.

Polymethyacrylate fluorimeter cuvettes (sigma C-0793)

Sep-pak c_{18} reverse phase cartridges (Waters) part no. 51910

Sterile plastic syringes (Becton Dickinson) Plastipak

Ultrafree-MC 10,000 NMWLfilter units (Millipore) (To process 400 µl volumes)

Western blotting apparatus (Biorad) Transblot cell

2.1:2 Enzymes

ACE (Sigma A-6778; rabbit lung) Stock is 1 µM in 2 mM TRIS-HCl, pH8.0, 100 mM NaCl, 10 µM EDTA

AP-N (Boehringer, 20 units/0.4 ml) (Stock solution is diluted 1/200 for assay)

MMPs Recombinant human progelatinase-A, progelatinase-B, procollagenase, prostromelysin and promatrilysin were purified from medium conditioned by the relevant transfected mouse myeloma cell line as described previously (Murphy *et al.*, 1992a; O'Connell *et al.*, 1994; Murphy *et al.*, 1992b; Kocklitis *et al.*, 1991; Crabbe *et al.*, 1992)

2.1:3 Substrates

ACE substrate (Sigma F-7131) N-(3-[2-furyl]acryloyl)-F-G-G. Stock solution is 1 mM in assay buffer

AP-N substrate L-leucine para-nitroanilide (Sigma) Stock is 20 mM in methanol/PBS (1:1 v:v)

Preparation of casein [^{125}I]-labelled β-casein is stored in 100 µl aliquots at -20°C. Prior to assay it may be purified by the addition of 20 µl of an 18% solution of TCA. Intact casein then forms a precipitate which may be isolated by centrifuging at 15,000 rpm for 10 minutes. The precipitate is dissolved in 0.5 ml of tetraborate buffer and then diluted to a final specific activity of 2 x 10^6 counts per ml.

collagen (type I prepared from rat skin). Acid soluble collagen was extracted and purified from rat skin as described by Cawston and Murphy, (1981). The final product is stored lyophilised at 4°C. The collagen content is determined

by amino-acid analysis and calculated from the hydroxproline content which makes up 13% of the total weight of collagen. Prior to assay collagen is dissolved at 1 mg/ml in 0.2 M acetic acid by slowly stirring for several hours at room temperature. The solubilsed collagen is then dialysed against collagen dialysis buffer for 16 - 32 hours. The pH of the final solution is retained at about pH 6.5 to prevent precipitation. For assay the pH is adjusted to 7.5 - 8.0 using 5 N sodium hydroxide.

[^{14}C]-labelled collagen Approximately 30 ml of unlabelled collagen (1 mg/ml) is dialysed against tetraborate buffer for 16 hrs. This is then transferred to a conical flask on ice. The total contents of 1 vial of [^{14}C]-acetic anhydride (250 μCi) are mixed with the collagen solution for 30 mins at 4°C. This is then dialysed for several days against 0.2 M acetic acid until the radioactivity in the dialysate has reached background levels. The radioactivity of the collagen is reduced to about 10^5 cpm/ml using unlabelled collagen solution and this is dialysed for 48 hrs in collagen dialysis buffer.

[^{14}C]-labelled gelatin [^{14}C]-labelled collagen (1 mg/ml) in 0.2 M acetic acid (above) is diluted into cold collagen (1mg/ml, 10^5 cpm/mg) in collagen dialysis buffer to give a final specific activity of 10^5 cpm/mg. The collagen is converted to gelatin by heating to 55 - 60°C for 60 minutes. The pH of this solution is then adjusted to pH 8 by the addition of a few drops of sodium hydroxide.

Heat appropriate mixture of collagen and [^{14}C]-labelled collagen in collagen dialysis buffer to 60°C for 1 hour. Store at 4°C. For assay adjust pH to pH 7.5 - 8.0.

Dnp-PLGLWAR (Stack and Gray, 1989) (Purchased from Nova biochem, A08563) E_{372} = 16,000 $M^{-1}cm^{-1}$. For a 25 mM solution add 25 mg to 1 ml of DMSO. Measure the absorbance at 372 nm and adjust the volume accordingly. Store in the dark at -20°C. For assay this stock solution is diluted to 4 mM with DMSO. Additional dilutions are carried out in peptide asay buffer. The final concentration of DMSO should be no greater than 0.5% (v/v) in the assay.

QF24 (Purchased from Dr. G.Knight, Strangeways research laboratory, Cambridge) E_{410} = 7,500 $M^{-1}cm^{-1}$. Dissolve 0.9 mg in I ml of DMSO. Measure the absorbance of a 1:10 solution (sample:assay buffer) at 410 nm. Adjust the volume with DMSO to give a stock concentration of 350 - 390 μM. Store in the dark at 4°C. For assay this solution is diluted 250 fold in peptide assay buffer.

2.1:4 Buffers

ACE assay buffer 50 mM HEPES, pH7.5, 0.3 M NaCl, 10 μM zinc chloride

AP-N assay buffer Sterile phosphate buffered saline (PBS)

Caseinase assay buffer 25 mM TRIS-HCl, pH 7.6, 10 mM $CaCl_2$, 0.05%Brij 35, 0.05% sodium azide (w/v)

Collagen dialysis buffer 50mM TRIS-HCl, pH 7.6, 200 mM NaCl, 0.003% sodium azide (w/v)

Diffuse fibril Assay buffer 100mM TRIS-HCl, pH 7.9 at r/t, 15 mM $CaCl_2$, 0.02% sodium azide (w/v)

Enzyme dilution buffer 50 mM TRIS-HCl, pH 7.9 at r/t, 0.02 sodium azide (w/v)

Gelatinase activation buffer 63 mM TRIS-HCl, pH 7.5, 65 mM NaCl, 10 mM $CaCl_2$, 0.025%Brij 35 (This is made up by combining equal volumes of peptide assay buffer and low salt enzyme buffer).

Low salt enzyme buffer 26 mM TRIS-HCl, pH 7.5, 30 mM NaCl, 10 mM $CaCl_2$,

Peptide assay buffer 100 mM TRIS-HCl, pH 7.5 at r/t, 100 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij 35 (v/v)

Pleural cavity lavage buffer PBS containing 50 mM EDTA

SDS-PAGE sample buffer (x5 concentrated) 2.5 M Tris-HCl, pH 6.8 (3.75 ml), glycerol (15 ml), SDS (4.5 g), deinonised water (8.5 ml). Stored at 4°C and heated for use.

Solution phase assay buffer 100 mM TRIS-HCl, pH 7.9 at r/t, 250 mM NaCl, 30 mM $CaCl_2$, 750 mM glucose, 0.02 % sodium azide (w/v)

Solution phase enzyme dilution buffer 50 mM TRIS-HCl, pH 7.9 at r/t, 5 mM $CaCl_2$, 0.05 Brij 35, 0.02% sodium azide (w/v)

Stopping buffer 100 mM sodium acetate pH 4.0

Tetraborate buffer 10 mM disodium tetraborate, pH 9.5, 200 mM $CaCl_2$ $6.H_2O$

Western blotting transfer buffer 100 mM CAPS buffer pH 11 (22.13g/L) was stored as a x 10 concentrated solution. For use 300 ml of CAPS is mixed with 300 ml of methanol and the volume is adjusted to 3 L with deionised water.

Western blotting iow salt wash buffer 20 mM TRIS-HCl, pH 7.6, 0.9% NaCl (w/v), 0.05% triton X-100 (v/v).

Western blotting low salt blocking buffer Low salt wash buffer containing 0.5% casein hammersten (w/v) (BDH).

Western biotting high salt blocking buffer 20 mM Tris-HCl, pH 7.6, 1 M NaCl, 0.4% lauroyl sarcosine (w/v).

2.2: Enzyme assays

2.2:1 MMP activation

Prior to their use in assays all MMPs have to be activated. The following methods of activation were developed using human recombinant enzymes. However, enzymes from other sources may be activated in a similar manner.

(i) Interstitial procollagenase

Interstitial procollagenase (1 - 4 μM) is incubated for 45 minutes at 35°C together with TPCK-treated trypsin (5 μg/ml) and prostromelysin (4 μg/ml). At the end of the incubation period the trypsin activity is terminated by the addition of SBTI (50 μg/ml) or PMSF (1mM). The activated collagenase is commonly allowed to stand for at least 15 minutes at 23°C (room temperature) prior to assay. Although this solution may be stable for several days at 4°C fresh batches are generally prepared for each assay.

This process is also used for the activation of truncated procollagenase which lacks the C-terminal domain and for neutrophil procollagenase.

(ii) Prostromelysin

Prostromelysin (1 - 2 μM) is incubated with TPCK-treated trypsin (0.01 mg/ml) for 15 - 30 minutes at 23°C. The trypsin activity is then terminated with SBTI (0.1mg/ml). The activated enzyme is stable for at least a week at 4°C, although like collagenase, fresh reagent is generally prepared prior to assay.

(iii) Progelatinase-A

Progelatinase-A (1.6 μM) is incubated in the presence of APMA (1 mM) in peptide assay buffer which includes 5% DMSO (i.e. APMA:gelatinase solution 1:20 v/v) for 16 hours at 23°C. The incubation period may be shortened by omitting Brij 35 from the assay buffer. Generally the APMA is not removed prior

to assay and this solution is stable at 4°C for 3 - 4 weeks. Approximately 90% activation is achieved by this method.

(iv) Progelatinase-B

Progelatinase-B (2 μM) is incubated in the presence of APMA (2 mM) in peptide assay buffer which includes 10% DMSO for 23 hours at 37°C. This solution is generally stored at -20°C prior to assay.

Alternatively, progelatinase-B (2 μM) may be activated by the addition of active stromelysin (0.17 μM) for 6 hours at 37°C. This produces a more stable species but the presence of stromelysin may interfere with the assay of inhibitors.

(v) Promatrilysin

Promatrilysin is activated by heating for 5 hours at 53°C (Crabbe *et al.*, 1992). This solution is stable for several weeks at 4°C.

2.2:2 Peptide assays for MMPs

The activity of MMPs is routinely measured using two fluoresecent peptides

1. Dinitrophenyl Pro-Leu-Gly-Leu-Trp-Ala- D-ArgNH_2 (Dnp-PLGLWAR; Stack and Gray, 1989).
2. 7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3 diaminopropionyl]-Ala-ArgNH_2 (Mca-PLGL Dpa AR; Knight *et al.*, 1992). This is referred to as QF24 throughout this study.

The fluorescence associated with tryptophan, W, (peptide 1) and Mca (peptide 2) is quenched by the presence of Dnp and Dpa, respectively. Cleavage of the G-L bond separates the two groups resulting in a corresponding increase in fluorescence. The rate of fluorescence increase (FU/min) is proportional to the rate of enzymic reaction.

(i) Dnp-PLGLWAR assay

Dnp-PLGLWAR assays are generally performed using a substrate concentration of 20 μM in 0.1M Tris-HCl, pH 7.5, containing 0.1M NaCl, 0.01M $CaCl_2$, 0.05% Brij 35 (peptide assay buffer) and 0.5% DMSO (Crabbe *et al.*, 1992). Assays are routinely conducted at 37°C for all the MMPs except gelatinase-A, which is assayed at 26°C due to its instability at the higher

temperature. Hydrolysis of the G-L bond is followed fluorometrically by measuring the increase in tryptophan (W) fluorescence (λ_{ex} 280, λ_{em} 346) using a Perkin Elmer LS 30 luminescence spectrometer.

A stopped assay is commonly employed, in which 20 µl of the reaction mixture is removed to 0.78 ml stopping buffer. Samples are then placed in an autosampler and the fluorescence measured using a flow cell. Fluorimeter settings are as follows;

Excitation wavelength	:280 nm
Emission wavelength	:346 nm
Monochromator scan mode	: Emission
Ordinate mode	: Fluorescence
Scale factor	: 1
Response	: 2
Abscissa mode	: Scan
Sample time / sec	: 5
Delay / sec	: 3
Purge time	: 0

The data is displayed using an LS 30 handler programme.

(ii) QF24 assay

MMP activity is measured using the substrate QF24 (1.4 -1.6 µM) in a total reaction volume of 2.5 ml of peptide assay buffer. Hydrolysis of the G-L bond is followed fluorometrically by measuring the increase in Mca fluorescence (λ_{ex} 328, λ_{em} 393) associated with the removal of the quenching Dpa group (Knight *et al.*, 1992). Fluorescence is monitored continuously using a Perkin Elmer LS 5B luminescence spectrometer with slit widths set at 5 mm. Rates are graphically displayed using the FLUSYS programme (Rawlings and Barrett, 1990). Reactions are calibrated at the start of each experiment using Mca-P-L (10 nM). The fluorimeter set-up is programmed as follows;

Excitation wavelength	:328
Emission wavelength	:393
Autozero	:Yes
Autoconcentration	:Yes (factor 100)
Response time	:3

Generally, 10 µl of substrate solution in DMSO is mixed with 2.465 ml of buffer in a quartz or polymethacrylate cuvette (Sigma) suitable for fluorescent measurements. The total volume is then adjusted to 2.5 ml with enzyme solution (25 µl). In the absence of a temperature controlled sample holder reactions are carried out at room temperature (22 - 25 °C).

2.2:3 Determination of K_m and k_{cat}/K_m values

The Michaelis constant (K_m) is the dissociation constant for the enzyme-substrate complex and it refers to the substrate concentration required to produce half-maximal velocity (V_{max}). It is experimentally determined by measuring enzymic rates in the presence of a range of substrate concentrations, ideally of the order of 0.1 - 10 times the K_m value. The enzfitter programme (Michaelis-Menten kinetics) can be used to determine the K_m from a plot of substrate concentration (x-axis) *versus* rate (y-axis). The insolubility of QF24 and fluorescence quenching at higher concentrations precluded an accurate estimation of K_m for any MMP although a lower limit can be established. Similarly, because of the insolubility of the Dnp-PLGLWAR substrate, accurate K_m values were only determined for the gelatinases.

The k_{cat}/K_m is a useful term for comparing the specificity of an enzyme towards a substrate and is an indication of the overall efficiency of an enzyme. It may be derived from the standard Michaelis-Menten equation;

$$v_i = \frac{V_{max}.\ S}{K_m + S}$$

where v_i is the initial rate and S is the substrate concentration. When S is not substantially lower than K_m the value for k_{cat}/K_m cannot be determined directly. Under conditions of $S << K_m$, however, the denominator, S, term becomes insignificant and the equation may be rearranged to give,

$$\frac{V_{max}}{K_m} = \frac{v_i}{S}$$

Since V_{max} is equivalent to the product of k_{cat} and the enzyme concentration [E] this becomes;

$$\frac{k_{cat}}{K_m} = \frac{v_i}{S\ .\ [E]}$$

The k_{cat}/K_m may, therefore, be determined directly using a substrate concentration at least 10 fold lower than the K_m. On occasions when this condition cannot be satisfied the K_m may be determined using the V_{max} value.

Since, the k_{cat}/K_m is often expressed in units of $M^{-1}s^{-1}$, rates such as FU/min, must be converted into substrate concentration terms. This conversion is routinely carried out for the QF24 assay during the process of calibration. Dnp-PLGLWAR fluorescence may be calibrated using a range of tryptophan concentrations. The precise relationship between fluorescence units and the exposure of tryptophan, changes according to the age of the fluorimeter lamp, but it is commonly in the range of 1 FU = 0.005 - 0.006 μM.

2.2:4 Determination of Ki values

(i) Dnp-PLGLWAR assay

Preparation of inhibitor

Inhibitors are commonly dissolved in methanol at a concentration of 10 mM. Prior to assay, samples are diluted 100 fold in methanol after which further dilutions may be performed using assay buffer. Between eight and ten inhibitor concentrations (double-dilutions) are frequently tested. Ideally, the Ki should lie close to the middle concentration. A suitable concentration range may be established by performing a preliminary "siter" assay using four inhibitor concentrations, for example 1, 10, 100 and 1000 nM. Except in exceptional circumstances (such as the measurement of very weak inhibitors) the final concentration of methanol should be lower than 1% of the total assay volume.

Method

All MMP inhibitors are initially screened with the Dnp-PLGLWAR substrate using assay conditions described previously. The final assay mixture contains enzyme (12 μl), inhibitor (12 μl), substrate (10 μl) and is adjusted to 120 μl with peptide assay buffer.

Briefly, gelatinase-A (0.05 nM), gelatinase-B (0.04 nM), stromelysin (3 nM), collagenase (2.5 nM) or matrilysin (0.25 nM) and a range of inhibitor concentrations (0.1 - 50 x Ki) are incubated for 1 - 4 hours at 23°C in peptide assay buffer. The reaction is started by the addition of substrate to a final concentration of 20 μM. After 16 hours at an appropriate temperature the reaction is terminated by removing 20 μl and adding this to stopping buffer and measuring the resulting fluorescence.

Apparent Ki (Ki_{app}) values are determined using Enzfitter (Leatherbarrow, 1987) by a non-linear regression of the following equation (Morrison and Walsh, 1988) for tight-binding, reversible inhibition;

$$v_s = \left(\frac{v_o}{2E_o}\right)\left[\sqrt{(Ki_{app} + I_t + E_t)^2 + 4E_t Ki_{app}} - (Ki_{app} + I_t + E_t)\right]$$

where v_o is the initial rate in the absence of inhibitor, v_s is the initial rate in the presence of inhibitor, E_t is the total enzyme concentration and I_t the total inhibitor concentration in the reaction mixture. The enzfitter programme for high affinity inhibition is essentially as follows;

Equation Name
High affinity inhibition

Y Axis Name	X Axis Name	VAR
Initial rate	[Inhibitor]	I

	Variable Name	VAR		Variable Name	VAR
1	Inhibition constant	Ki	5		
2	Uninhibited rate	vo	6		
3			7		
4			8		

	Prompted Constant	VAR		Prompted Constant	VAR
1	Enzyme concentration	Eo	3		
2			4		

Equation Definition	
Temp1 Temp2	
Y =	(vo/(2*Eo))*((Eo-I-Ki)+SQRT(SQR(Ki+I-Eo)+4*Ki*Eo))

True Ki values may be determined using the relationship for competitive inhibition, $Ki_{app} = Ki\left(1 + {}^{S}/_{K_m}\right)$. Collagenase, stromelysin and matrilysin assays are performed under first order conditions of S << K_m and, therefore, Ki_{app} approaches the true Ki. Substitution of the appropriate K_m value in the above equation permits the Ki values of inhibitors of gelatinase-A and gelatinase-B to be calculated.

(ii) QF24 assay

The Dnp-PLGLWAR assay cannot be used to determine the potency of inhibitors with Ki values lower than 100 pM against gelatinase-A. In such cases, the QF24 assay is commonly used as a secondary screen, since the superior

sensitivity of this substrate (chapter 3) permits a reduction in the concentration of the enzyme.

Method

Gelatinase-A (20 pM) and a range of inhibitor concentrations (0.1 - 50 x Ki) are incubated in a volume of 2.49 ml of peptide assay buffer for 1 - 4 hours at 23°C. The reaction is started by the addition of substrate (10 µl) to a final concentration of about 1.4 µM. Hydrolysis rates are then determined over the first 1 - 2 minutes of the reaction using the flusys programme (Rawlings and Barrett, 1990). Ki_{app} is calculated as described above. The substrate concentration of 1.4 µM is presumed to be significantly lower than the K_m and, therefore, the Ki_{app} approximates to the Ki.

2.2:5 Titration of enzymic activity

The concentration of active MMP can be determined by titrating its activity against a high affinity inhibitor that binds to the enzyme with a 1:1 stoichiometry, under conditions in which the dissociation of the enzyme-inhibitor complex is negligible. TIMPs which are the natural inhibitors of MMPs were often used for this purpose. Whilst TIMP-1 is the most commonly employed titrant, TIMP-2 is preferable for the determination of gelatinase-B activity, since TIMP-1 can bind to the C-terminal domain of its proenzyme form. The concentrations of TIMP-1 and TIMP-2 were determined by titration against a standard solution of stromelysin, as described by Willenbrock *et al.*, (1993).

Method

Titrations are performed using an enzyme concentration of 20 - 50 nM and TIMP concentrations in the range of 2.5 - 100 nM. Enzyme and inhibitor are incubated at room temperature in a total volume of 50 µl of peptide assay buffer. After 1 - 2 hours aliquots are removed and then diluted appropriately for assay against either of the two fluorescent peptide substrates. Dnp-PLGLWAR assays are routinely performed at 37°C (except gelatinase-A) and samples are taken at intervals up to 30 minutes and analysed as described above. QF24 assays are performed at room temperature with continuous monitoring of the hydrolysis rate. In both cases only the linear portion of the progress curve is used, to avoid effects due to the dissociation of the TIMP-enzyme complex.

The active enzyme concentration is determined form the x-axis intercept of a plot of the initial rate of substrate hydrolysis (y-axis) *versus* TIMP concentration (x-axis).

2.2:6 High molecular mass substrate assays

The following high molecular substrate assays (diffuse fibril, soluble collagen, gelatin , casein) are all based on differential centrifugation. In each case soluble radiolabelled material, formed as a result of enzyme action, is separated from uncleaved substrate by centrifugation. The amount of radioactivity in the supernatant is proportional to enzymic activity.

(i) The determination of IC_{50} values using the diffuse-fibril assay for collagenase (Cawston and Barrett, 1979)

The collagenolytic activity of collagenase is commonly measured against collagen of three physical forms, namely fibrillar, semi-soluble (diffuse-fibril) or soluble (reviewed in Cawston and Murphy, 1981). The former, which uses preformed fibrils is closest to mimicking the natural substrate but it is limited by the poor specific activity of collagenase. Collagenase is most active against soluble collagen but artifacts may be introduced by the solubilising agent. The most frequently employed assay is the diffuse-fibril type since it incorporates properties of both the other assays.

The IC_{50} value is the concentration of inhibitor required to inhibit enzymic activity by 50%. It is calculated by plotting the Log_{10} inhibitor concentration (x-axis) *versus* percentage inhibition of the activity of an enzyme control. The IC_{50} may then be determined manually from the resulting sigmoidal plot or using an appropriate enzfitter programme.

The following controls should also be included,

1. No enzyme blank (This value is subtracted from all other results)
2. Enzyme without inhibitor (Eo) (This represents 0% inhibition)
3. Total digestion by bacterial collagenase (This gives an indication as to whether (2) is on the linear portion of the concentration-activity curve)

Method

A range of inhibitor concentrations are prepared based upon the criteria described for Ki assays. Assays (triplicate) are then performed in a total volume of 300 µl using 0.4 ml eppendorf tubes as follows;

Inhibitor in a volume of 10 µl of methanol or DMSO is added to 70 µl of enzyme dilution buffer. The volume is then adjusted to 200 µl with 100 µl of collagenase assay buffer and 20 µl of activated collagenase (100 nM). This solution is allowed to stand at room temperature for 30 - 60 mins. The reaction is started by adding 100 µl [^{14}C]-acetylated collagen (1mg/ml, 10^5 cpm/mg). After whirli-mixing, samples are incubated for a further 100 mins at 35°C. The tubes are flicked in order to cause the collagen to condense into a ball of fibrils. They are then centrifuged for at 12,000 rpm for 10 minutes to precipitate the intact collagen. 100 µl of the supernatant is then immediately removed and mixed with 3 ml of an aqueous compatible scintillation fluid. Cpms are counted using the ^{14}C-channel of a scintillation counter (eg. Packard)

(ii) The solution phase assay for collagenase

The property of collagen to form insoluble fibrils at neutral pH at concentrations in excess of 100 µg/ml precludes the use of the diffuse fibril assay for obtaining kinetic parameters by classical enzymological methods. Fibril formation may, however, be inhibited by the inclusion of glucose so that assays can be performed in a "total-solution-phase" system. The procedure described here is based on an assay method first developed by Terato *et al.* (1976). Assays (triplicate) are performed in a total volume of 300 µl in 1 ml eppendorf tubes.

Method

Collagenase made up in 100 µl of enzyme dilution buffer is added to 100 µl of glucose assay buffer. The temperature of the reaction mixture is then raised to 35°C prior to the addition of 100 µl of [^{14}C]-labelled collagen (1 mg/ml, 10^5 cpm/ml). The reaction proceeds for 60 minutes at 35°C and then enzymic activity is quenched by the addition of 15 µl of 1,10-phenanthroline. Samples are then allowed to stand at 35°C for a further 60 minutes to encourage unfolding of the cleaved collagen. After cooling to room temperature, intact collagen is precipitated by the addition of an equal volume of dioxane solution followed by centrifugation in a microfuge (about 12,000 rpm) for 10 minutes. Radioactivity in the supernatant is measured by removing 400 µl to 4 ml of scintillation fluid and

counting on a ^{14}C channel. Control assays are similar to those described for the diffuse fibril assay.

(iii) Gelatin assay for gelatinase

Denatured fibrillar collagen is thought to be a physiological substrate for members of the gelatinase family. Whilst, *in vivo*, it is likely to be produced as a result of collagenase action, gelatin may be formed artificially by incubating type I collagen at elevated temperatures. The following procedure is based on the method described by Sellers *et al.* (1978).

Method

[^{14}C]-labelled gelatin substrate is prepared as described in section 2.1:3. A range of inhibitor concentrations are prepared by doubling dilution as described for Ki determination. Assays (triplicate) are then performed in a total volume of 300 µl using 1 ml eppendorf tubes as follows;

Gelatinase-A (0.5 nM) and a range of inhibitor concentrations are incubated for 2 hours in a final volume of 200 µl peptide assay buffer. Enzyme only (Eo), blank and total hydrolysis samples are treated similarly. The reaction is started by the addition of [^{14}C]-gelatin (100 µl). After 100 minutes at 26°C the enzymic activity is terminated by the addition of 60 µl of 90% solution of TCA. Prior to processing, samples are stored at 4°C overnight or on ice for 1 hour. The tubes are subsequently centrifuged at 12,000 rpm for 10 minutes at 4°C to precipitate the uncleaved gelatin. 200 µl of supernatant is then added to 3ml of scintillation fluid and the radioactivity is measured on the [^{14}C] channel of a Packard scintillation counter or equivalent instrument. IC_{50} values are determined as described for the diffuse fibril assay.

In a variation on the above procedure the incubation time may be increased to 16 hours to permit the gelatinase concentration to be decreased. This assay is limited, however, by an increase in the level of cpm in the blank supernatant.

(iv) Caseinase assay for stromelysin (based on Cawston *et al.* (1981)

The hydrolysis of casein is commonly employed for the detection of non-specific proteinase activity. Whilst all MMPs tested, possess some caseinase activity (collagenase has poor activity) it is most frequently used for the measurement of stromelysin.

Method

[^{125}I]-labelled β-casein substrate is prepared for assay as described in section 2.1:3. A range of inhibitor solutions are prepared as described above. Assays (triplicate) are performed using stromelysin at a final concentration of 30 - 35 nM in a total volume of 100 µl in 0.4 ml eppendorf tubes as follows;

Stromelysin (20 µl) is added to a solution containing inhibitor (5 µl) mixed with 65 µl of caseinase assay buffer. Samples are incubated for 60 minutes at room temperature prior to the addition of 10 µl of radiolabelled casein substrate to initiate the reaction. After 60 minutes at 37°C the reaction is terminated by adding 20 µi of unlabelled carrier β-casein (1mg/ml) followed by a solution of 18% TCA (24 µl). Samples are whirli-mixed and then placed on ice for 60 minutes. Undigested casein is precipitated by centrifugation at 15,000 rpm, for 15 minutes at 4°C. 100 µl of the supernatant is then placed in an LP4 tube and counted using an LKB gamma counter. IC_{50} values are determined as described previously.

2.2:7 Other assays

(i) Peptidyl-dipeptidase A

Inhibitors were tested against ACE using the substrate furylacroyl-F-G-G essentially as described by Bunning *et al.* (1983). Enzymic activity is followed by measuring the decrease in absorbance at a wavelength of 328 nm using an HP 8452A spectrophotometer.

Prior to assay, the enzyme, which is stored with EDTA is incubated in ACE assay buffer at room temperature for 60 minutes.

Method

For assay, ACE (20 nM) is mixed with inhibitor (100 µM) in a total volume of 0.9 ml of assay buffer in a 1 ml quartz cuvette. The reaction is started by the addition of 100 µl of substrate (final concentration 100 µM) and the resulting decrease in absorbance is measured using a 1 cm light path. Control samples,

including enzyme without inhibitor and an inhibitor vehicle blank, are scanned simultaneously. Reactions are monitored for 20 minutes and then the initial rates (AU/min) are established from the linear part of the progress curve (1 - 6 minutes).

(ii) Aminopeptidase-N (AP-N)

Inhibition of AP-N activity was tested using the substrate L-leucine p-nitroanilide as described by Tieku and Hooper (1992). Hydrolysis of the substrate causes the release of the p-nitroanilide group which results in an increase in absorbance at 405 nm. Assays are performed in 96 well microtitre plates (NUNC) using a UVmax microtitre plate reader.

Method

Inhibitor is dissolved in methanol to a concentration of 1 mM. This solution is then diluted appropriately (doubling dilutions) to provide a range of concentrations giving 0 -100% inhibition in the assay (see IC_{50} determination).

Inhibitor solution (10 µl) is mixed with 170 µl of PBS in a microtitre plate prior to the addition of 10 µl of enzyme. After 1 hour at 37°C the reaction is started by adding 10 µl of substrate to give a final concentration of 1 mM. Reaction rates may be followed using the soft max programme set to the following parameters.

MODE:	kinetics	AUTOMIX:	ON
Wavelength:	405	RUN TIME:	15:00 (mins)
CALIBRATION:	ON	LAG TIME:	0
OD LIMIT	2.2		

Initial rates (mOD/min) are determined from the linear portion of the progress curve and IC_{50} values may be determined as described previously.

2.3: Protein characterisation

2.3:1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was routinely performed using precast 10 - 20% polyacrylamide gels purchased from Daiichi Pure Chemical company Ltd., Tokyo, Japan. Samples to be analysed typically contained 1 µg of protein and were prepared by boiling for three minutes in standard sample buffer (Laemnli, 1970) which included 2% (v:v) β-mercaptoethanol for reducing conditions. Samples

were typically electrophoresed at a constant current of 35 - 40 mA until the dye front reached the bottom of the gel. Completed gels were stained for protein by placing them in Coomassie-blue R-250 stain for 1 hour. Gels were then destained using a solution of 7.5% (v/v) acetic acid or 10% (v/v) acetic acid and 40% (v/v) methanol. Molecular masses (kDa) were generally estimated using a range of molecular mass standards purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

2.3:2 Electroblotting and N-terminal sequencing

Protein samples were N-terminally sequenced either directly or after electroblotting as described by Crabbe *et al.* (1992). In the latter method the sample is first subjected to SDS-PAGE according to the method of Hunkerpiller (1983). Protein is then electroblotted onto a poly(vinylidene) membrane (Matsudaira, 1987) and stained using 0.1% (w/v) Ponceau S dye in 1% (v/v) aqueous acetic acid (Aebersold *et al.*, 1987) and the relevant band is then excised. The blotted sample is applied to Bioprene-treated glass fibre discs which are loaded onto an Applied Biosystems 470A gas-phase sequencer and N-terminally sequenced by Edman degradation. The resulting phenylthiohydantoin-derivatised amino-acids are analysed with an Applied Biosystems 120A HPLC.

2.3:3 Western blotting

Western blotting was used to identify the collagenase domain recognised by the monoclonal antibody, MAC065.

Transfer

Samples were loading symmetrically onto both halves of a 10 - 20% SDS-polyacrylamide gel and electrophoresed as described above. This allows one half of the gel to be electroblotted and the other half to be stained for protein. Proteins were transferred onto immobilon membranes by electroblotting (Biorad Trans-Blot apparatus) for 16 hours at a constant voltage of 30 V in transfer buffer.

Detection

The blotted membrane is soaked in casein blocking buffer at room temperature for 2 hours and then washed several times with low salt buffer and water. The membrane is then soaked for 3 hours at room temperature in 100 ml of the primary antibody (MAC065, 6 µg/ml) dissolved in low salt blocking buffer.

The washing process is then repeated. The washed membrane is placed on a glass plate and covered with 10 - 20 ml of the secondary antibody (horse radish peroxidase linked anti-mouse fc chain) in high salt blocking buffer and then incubated for 1 hour in a humidity chamber. After washing, bands are visualised by exposure to autoradiographic film (5 seconds) using the ECL detection system (Amersham).

2.4: Pharmacokinetic methods

2.4:1 Inhibitor extraction

An extraction step is required before measurement of inhibitor levels in blood. This is necessary because,

1. Plasma components interfere with UV monitoring
2. Plasma significantly raises the background fluorescence in enzyme assays
3. Plasma contains natural inhibitors of MMPs such as TIMP and α-2 macroglobulin

Several extraction procedures were developed for use with a range of inhibitors according to their individual physicochemical properties and the means of detection.

(i) Ethyl acetate extraction

Ethyl acetate extracts lipophilic substances from aqueous solutions. Generally, 0.5 ml of whole blood is mixed with 2 ml of ethyl acetate. After 1 - 4 hours of occasional mixing, 1 ml of the clear supernatant containing extracted inhibitor is removed to 2ml eppendorf tubes with perforated lids. The ethyl acetate is then removed by vacuum centrifugation (speed vac). The final yield is in the range of 80 - 100% depending on the physiochemical properties of the inhibitor.

(ii) Dichloromethane (DCM)

This reagent was used for the extraction of inhibitors with some degree of water solubility. The procedure is essentially similar to that described for ethyl acetate but differs in that the DCM phase is not the supernatant but lies below the aqueous phase. In order to remove a fraction of the organic phase the pipette must be applied through the plasma layer.

(iii) Soiid-phase extraction

Sep-pak c_{18} reverse phase cartridges were used as an alternative to organic extraction procedures. Their effectiveness resides in their ability to bind to both lipophilic and partially water soluble inhibitors which makes the technique available for the extraction of a wide range of inhibitors. Conversely, since they bind with equal avidity to various blood constituents the final extract is not as pure as that derived from organic extraction procedures. Consequently, it is commonly used in combination with ultrafiltration using ultrafree cartridges. Sep-pak extraction proceeds in the following steps. Solutions are applied to the end of the cartridge using a plastic syringe.

Step 1 Prepare plasma from whole blood by centrifugation at 4,000 rpm

Step 2 Wash the sep-pak cartridge with 10 ml of methanol

Step 3 Wash the cartridge with 2ml of aqueous 70% acetonitrile / 0.1% TFA (v/v)

Step 4 Wash the cartridge with 10 ml of 0.1% TFA (v/v)

Step 5 Apply the plasma sample

Step 6 Wash through unbound material with 10 ml of 0.1% TFA

Step 7 Elute bound inhibitor with 2 ml of aqueous 70% acetonitrile/0.1% TFA (v/v)

Step 8 Lyophilise the bound fraction by vacuum centrifugation

Step 9 Reconstitute in methanol and then dilute into an appropriate buffer

Yield of inhibitor is characteristically greater than 90%.

(iv) Methanol precipitation

Extraction procedures are optimised to maximise the yield of the parent compound which is invariably lipophilic. In order to do this effectively they must discriminate against more hydrophilic substances. This limits their capacity to isolate drug metabolites which are often more hydrophilic than the parent. Plasma proteins may be precipitated by organic solvents such as acetonitrile and methanol. These methods leave small molecules that are soluble in organic solvent in solution they are relatively non-specific and so an additional 'clean up' step may be required. Methanol precipitation is performed as follows overleaf;

Step 1 Incubate methanol on ice for 30 - 60 minutes

Step 2 Prepare plasma from whole blood by centrifugation at 4,000 rpm

Step 3 Mix 1 ml of ice-cold methanol with 0.25 ml of plasma sample

Step 4 Store sample on ice for 1 - 2 hours with occasional mixing

Step 5 Centrifuge sample in a microfuge for 10 minutes

Step 6 Remove 1ml of the supernatant

Step 7 Lyophilise supernatant by vacuum centrifugation or analyse directly

Step 8 Reconstitute lyophilised sample in methanol and then dilute into an appropriate buffer

Yield of inhibitor is 100% by this method.

2.4:2 Measurement of inhibitor leveis in biological fiuids

(i) Titration against gelatinase-A

This method is based on the observation that one molecule of inhibitor binds to one molecule of gelatinase-A. The high specific activity of gelatinase-A towards the QF24 substrate makes this assay very sensitive, since low concentrations of enzyme and hence inhibitor may be tested. There are, however, two major limitations to its use, namely; (1) The Ki of the test compound must be low enough such that dissociation of the enzyme-inhibitor complex is negligible. In practice this limits the technique to sub-nanomolar inhibitors and (2) The assay cannot discriminate between parent compound and active metabolites.

Construction of the titration curve

The linearity between the reduction in enzymic rate and inhibitor concentration may be established from the titration curve which is constructed as follows.

Briefly, 50 µl of active gelatinase-A (20 nM) is mixed with 50 µl of inhibitor at concentrations of 0, 2.5, 5, 7.5, 10 ,15, 20, 30 and 40 nM in peptide assay buffer (inhibitors diluted from methanol). After 2 hours at room temperature 25 µl of sample is added to 2.475 ml of peptide assay buffer and the reaction is started by adding 10 µl of QF24 (1.4 -1.6 µM). Hydrolysis of QF24 is monitored as described previously for the titration of TIMP (section 2.3.5).

By plotting initial rate (FU/min) on the x-axis *versus* inhibitor concentration (nM) on the Y-axis, the value (V) of an unknown inhibitor concentration may be determined directly using the enzfitter programme (linear regression).

This method may be modified according to the extraction procedure. The blood concentration of inhibitors extracted into ethyl acetate, for example, is calculated from a titration curve of ethyl acetate extracted inhibitor.

Procedure for the determination of inhibitor levels in blood after *in vivo* experiments

Extracted inhibitor from the blood of 5 animals representing a single time-point is pooled and then titrated at three concentrations as described above. This establishes an approximate concentration for a more detailed study. Extracts from the blood of individual animals are then tested at appropriate dilutions. The inhibitor concentration can be established from the corresponding rate on the titration curve. The concentration of inhibitor in the original blood sample may then be calculated by multiplying this figure by the appropriate dilution factor.

(ii) Capillary electrophoresis (CE)

Capillary electrophoresis separates charged compounds in a small diameter capillary filled with buffer. The separated peaks may be detected by several methods including mass spectrometry but most commonly an integrated flow cell is used with UV detection. The technique combines high resolving power with a sensitivity that is superior to HPLC. The source of this sensitivity, however, is the ability of the technique to measure very small amounts (nl) of concentrated sample. This has limited its application in pharmacokinetic studies which often involves dilute sample solutions This problem was addressed by manipulating the procedure by which sample was loaded (Method 1). For more concentrated samples a standard procedure was employed (Method 2).

Method 1 (dilute sample)

Electrokinetic loading effectively concentrates sample ions on the capillary by removing them from the solution. It is limited, however, by a comparative lack of reproducibility which can be overcome by the inclusion of a range of spiked controls. This method is suitable for inhibitors such as CT543 (Fig. 5.1) which possess a positively charged group. Since the sample is loaded electrokinetically competing charged ions should first be removed. This may be achieved by extracting the compound with an organic solvent or sep-pak and then reconstituting the lyophilised extract in methanol. The methanolic solution is then

passed through an ultrafree filtration unit as a final clean up step. The sample is analysed by CE using the following conditions.

CAPILLARY	Fused silica, 3 mm pathlength, 75 μm I.D., length to flow cell 80 cm, total length 110 cm
SAMPLE SOLVENT	Methanol
RUNNING BUFFER	100 mM Sodium citrate pH 2.5
INJECTION	Electrokinetic, 30 seconds, 5 kV
RUN CONDITIONS	20 minutes, 20 kV, (+ve)
TEMPERATURE	30°C
DETECTION	210 nm

Peak areas were integrated using a trivector computer. The response is linear between 5 and 100ng loaded on the column.

Method 2 (concentrated sample)

Samples are generally applied to the CE capillary using vacuum loading. This method is reproducible since the rate of flow of sample liquid onto the column can easily be controlled. The concentration of the sample, however, is not altered by this method. It is applicable to the measurement of a range of charged inhibitors. The following conditions were applied to the measurement of the inhibitor, CT989 (Fig. 5.1).

CAPILLARY	As above
SAMPLE SOLVENT	50% Aqueous methanol
RUNNING BUFFER	20 mM TRIS-HCl pH 9.0, 28.8 mM borate,0.68 mM EDTA, 30% methanol (v/v)
INJECTION	Vacuum, 30 seconds
RUN CONDITIONS	20 minutes, 30 kV, (+ve), (current 4 mA)
TEMPERATURE	30°C
DETECTION	200 nm

Peak areas are integrated as described above. The response is linear between 1 and 20 μg/ml.

(iii) HPLC (concentrated sampie)

HPLC was used in this study for testing extraction efficiency, a process which may be performed using high concentrations of inhibitor. Samples, which typically contained greater than 100 µg of inhibitor, were analysed on a varian HPLC using a dynamax c_{18} reverse-phase column with detection at 254 nm.

Aqueous samples (500 µl) can be applied directly to the column although plasma cannot be applied without an extraction step. Organic samples are lyophilised and then reconstituted in 50 µl of methanol and adjusted to 1 ml with 0.1% aqueous TFA (v/v). Peaks are resolved using an 10 - 40% gradient of acetonitrile, at a flow rate of 1 ml/min, for 20 minutes.

2.4:3 Mouse pleural cavity assay

This assay derives from the rat pleural cavity assay described by Lark *et al.* (1990) in which stromelysin and [^{3}H]-transferrin were co-injected into the rat pleural cavity. The enzymic activity of stromelysin on this substrate was shown to be inhibited by the administration of TIMP-1. The assay described here is modified to study the inhibition of gelatinase-A by low molecular mass inhibitors. It is essentially a variation on the *in vitro* gelatin degrading assay reported in section 2.2:6

Preliminary experiments were undertaken to ensure that (1) the activity of the enzyme control was on the linear part of the concentration-rate curve and (2) that there was no significant loss of gelatin or its fragments from the pleural cavity during the time-course of the assay.

Method

200 µl of [^{14}C]-labelled gelatin (1 mg/ml) with a specific activity of 100,000 cpm/mg is mixed with an equal volume of active gelatinase-A (20 nM) in peptide assay buffer. The mixture is cooled on ice before injection into the pleural cavity of an anaesthetised mouse. A needle of sufficient length as to prevent damage leading to internal bleeding was used for this purpose.

After 30 minutes, the mouse is sacrificed and the cavity is then aspirated and lavaged using 1 ml of PBS containing 50 mM EDTA. Intact gelatin is precipitated by adding TCA to a final concentration of 15% (v/v). Samples are then processed as described in section 2.2:6. Radioactivity in the supernatant is proportional to the level of enzymic activity and the presence of inhibitor depresses this value in a concentration dependent manner.

By administering inhibitor at a pre-determined time before the injection of substrate and enzyme, the assay can be used to screen for inhibitors with superior pharmacokinetic properties. The study can be extended by dosing at a range of times prior to assay to produce an inhibition time-course. Variation of the route of administration permits the study of bioavailability. The assay cannot, however, be used to determine inhibitor concentrations since the dependency of inhibition on inhibitor concentration is affected by the tightness of inhibitor-enzyme binding. It is, therefore, most effective for the comparison of compounds with similar potencies.

2.4:4 Determination of pharmacokinetic parameters

(i) Systemic haif-life ($t_{1/2}$)

Following the rapid intravenous administration of a compound there is an early distribution phase, during which the compound is distributed to the tissues followed by an interval during which it is eliminated from the body (elimination phase). The simplest model to describe this process assumes that the body acts as a single compartment throughout which the compound is distributed homogeneously.

Many compounds exhibit more complex behaviour which can only be described using more than one compartment. Throughout this study data was analysed using a 2-compartmental model. In this model, the body is represented by two compartments, a central one which comprises the blood and easily accessible tissues and a peripheral one into which compound distributes more slowly.

The decline in plasma concentration of the drug is biexponential. Accordingly, the concentration can be described by the sum of two monoexponential terms,

$$C = A \cdot e^{-\alpha . t} + B \cdot e^{-\beta . t}$$

where A and B are the intercepts of the respective monoexponential curves on the y-axis, $A + B = C_0$ (concentration at time 0) and α and β are the hybrid rate constants equal to the slopes of the Ln concentration-time curve of the two monoexponential terms.

The determination of the β-phase half-life ($t_{1/2}$ {β}) is particularly useful for the study of MMP inhibitors. Experimentally it is determined by measuring the plasma levels of inhibitor after intravenous dosing. A plot of Log_{10} concentration

(y-axis) *versus* time (x-axis) comprises a relative steep α-phase which is then followed by a more shallow β-phase. The slope of this second phase, also known as the terminal phase equals $-\beta / 2.303$ and the corresponding $t_{1/2} = 0.693 / \beta$. The $t_{1/2}$ value was determined both manually and by using the Siphar pharmacokinetic programme.

(ii) Oral bioavailability

This is a measure of the relative amounts of drug appearing in the plasma after oral (p.o.) and intravenous administration (i.v.). The latter is presumed to be 100% bioavailable. Oral bioavailability may be calculated from the ratio of the area under the plasma concentration-time curves (AUCs) for the two routes of administration. AUCs may be determined manually using the trapezoidal method as shown in the following hypothetical example.

n	Time (mins)	Concentration (μg/ml)	(A) $Time_{n-(n-1)}$	(B) $Cn + C(n-1)_{/2}$	(A) x (B) (μgmin/ml)
1	0	0	-	-	
2	1	40	1	20	20
3	5	20	4	30	120
4	10	15	5	17.5	87.5
5	30	5	20	10	200
6	60	2	30	3.5	105

AUC = 532.5 μgmin/ml

Plasma concentrations (C_n) are determined at various times (T_n) after administration of the compound. The AUC is most accurately extrapolated to infinity, however, this was not generally applicable to the analysis of oral data since the concentration-time curves were often very flat. Consequently, AUC values were generally determined to the last time-point as presented above. Values were also calculated by the Siphar pharmacokinetic programme.

Chapter 3

CHAPTER 3

CHARACTERISATION OF HUMAN RECOMBINANT GELATINASE-A AND GELATINASE-B

3.1: Introduction

This Chapter describes the biochemical properties of gelatinase-A and gelatinase-B. The study focuses on three important features of these enzymes;

1. Activation of the proenzyme
2. The contribution of the C-terminal domain to enzyme action
3. The specific activity of the activated enzyme

For an MMP to become catalytically competent it must first undergo a process of activation. As described earlier, (Chapter 1.3:3) the end-point of the activation process is the removal of about 80 amino-acids from the N-terminus of the proenzyme with a concomitant reduction in molecular mass of 10 kDa as visualised by SDS-PAGE under reducing conditions (reviewed in Birkedal-Hansen, 1993). The events leading to the eventual activation of an MMP may provide an additional level at which to target therapeutic agents but the mechanism is still poorly understood.

Progelatinase-A is comparatively resistant to hydrolysis by serine proteinases such as trypsin and neutrophil elastase (Okada *et al.*, 1990; 1989b). The primary sequence of the propeptide shows that it lacks the basic amino-acid rich 'bait region' of other MMPs that makes them a substrate for trypsin-like proteinases (Chapter 1 (1.3:3). Artificial activation can, however, be affected by a variety of agents of which APMA is most commonly used. This agent causes the gelatinase to remove its own propeptide in a process which is thought to be initiated by an intramolecular cleavage (Okada *et al.*, 1990). At what stage intermolecular cleavages may also contribute to this process is still unclear. As yet no physiological counterpart to APMA has been identified and although activation by a proteinase is strongly suspected there has been little progress in discovering a suitable candidate.

Evidence for a cellular involvement has been presented by several laboratories (Brown *et al.*, 1990; Overall and Sodek, 1990; Ward *et al.*, 1991; Emonard *et al.*, 1992; Monsky *et al.*, 1993). Progelatinase-A can, for example, be activated in the presence of appropriately stimulated intact cells or cell

membranes (Ward *et al.*, 1991). Moreover, this reaction can be inhibited effectively by TIMP-2 and more poorly by TIMP-1. More recently, Strongin *et al.* (1993) identified an intermediate produced by the action of a stimulated human fibrosarcoma cell-line (HT1080) on progelatinase-A. By showing that activation could be competitively inhibited by a 26 kDa peptide derived from the C-terminus they were also able to demonstrate a possible involvement of the C-terminal domain.

The following studies are specifically aimed towards (1) characterising the sequence of events leading to APMA activation and (2) identifying a proteinase which fulfils the requirements for being considered as a physiological activator of progelatinase-A.

The role of the C-terminal domain in activation was studied by using a C-terminally truncated gelatinase (NGL-A). Finally, the specific activity defined by the k_{cat}/K_m was determined against several substrates in order to determine suitable enzyme concentrations for kinetic studies (Chapters 5 and 6).

The activation of progelatinase-B has been studied by a number of groups but the data emerging has been somewhat conflicting. Desrivieres *et al.* (1993) reported that tissue kallikrein, a component of the inflammatory response, efficiently activated progelatinase-B making it a candidate for the role of *in vivo* activator. In contrast a closely related enzyme, plasma kallikrein, was unable to carry out the activation. Progelatinase-B can, like neutrophil procollagenase (Knauper *et al.*, 1993), be activated directly by stromelysin (Ogata *et al.*, 1992). Ogata and his co-workers showed that stromelysin hydrolyses the bond between R^{87} - F^{88} resulting in a drop in molecular mass from 92 kDa to 82 kDa. In contrast Okada *et al.* (1992) observed active species of 68 kDa with APMA and 64 kDa with stromelysin treatment. Other groups have found that the C-terminal domain is also lost during this process and indeed Goldberg *et al.* (1992) presented evidence that removal of this domain is a prerequisite for the efficient activation of progelatinase-B by stromelysin. In other studies, Triebel *et al.* (1992) reported that APMA produces an enzyme which is fully active despite retaining the cysteine involved in maintaining latency. All these studies use a gelatin substrate to follow activation but this makes a kinetic treatment of the data difficult (Chapter 6).

In this work, activation processes were investigated using SDS-PAGE and N-terminal sequencing to identify the products of activation. The k_{cat}/K_m

values of the active forms were then measured against QF24 (Knight *et al.*, 1992). Additionally, the rates at which recombinant human progelatinase-B was activated by several agents were compared and the influence of the C-terminal domain on this process was assessed.

3.2 : Results

3.2:1 Progelatinase-A activation

(i) Time-course of APMA activation

Progelatinase-A can be conveniently activated in the presence of the universal MMP activator, APMA. The time-dependent generation of mature enzyme was followed using a peptide substrate and the identity of the fully activated species was determined by N-terminal sequencing.

Progelatinase-A (1μM) was incubated at 23°C with APMA (1mM). At times ranging from 0 - 24 hours (hrs). samples were removed for assay against QF24 (Fig. 3.1). The rate of enzyme activity was determined by linear regression analysis. Progelatinase-A is maximally activated after 8 hrs under these conditions. Previous work has indicated that the rate of activation is buffer dependent as changes in the level of Brij 35 or ionic strength can have significant effects on the time taken to reach maximum activity (data not shown). Rates of APMA activation can, therefore, only be compared using identical conditions.

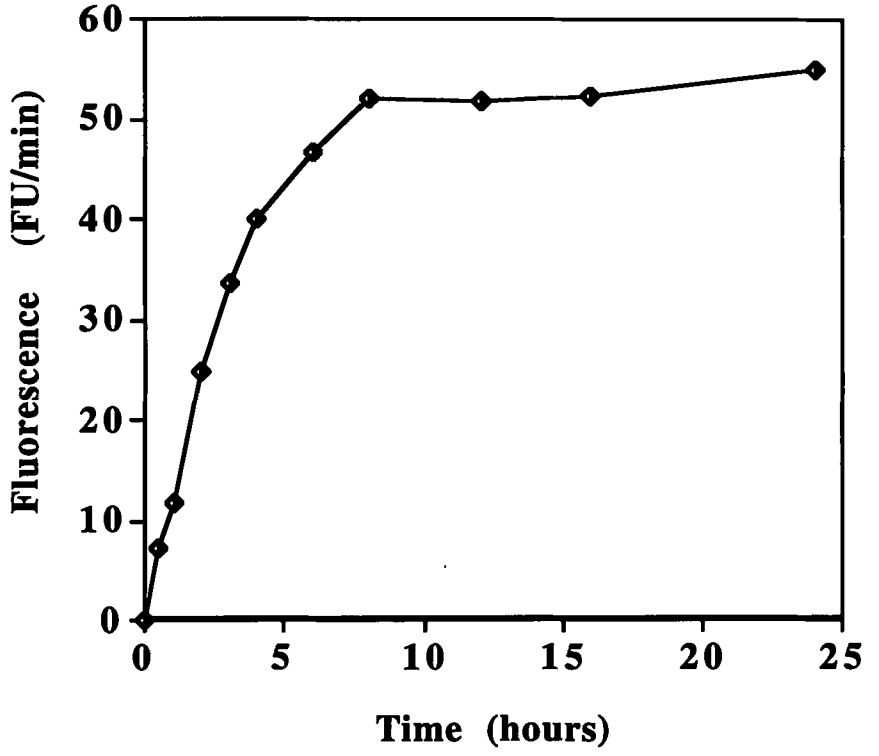
In a further experiment, progelatinase-A was incubated for 16 hrs in the presence of APMA. The sample was then analysed using SDS-PAGE. APMA caused a reduction in molecular mass from 72 kDa to 66 kDa (Fig. 3.2). N-terminal sequencing of the 66 kDa activated form identified a single N-terminus corresponding to Y^{81}.

This activation procedure was subsequently used to generate a single form of the enzyme for all kinetic studies of the enzyme and its inhibitors.

(ii) Effect of TIMP-1 and a synthetic Inhibitor CT989 on the activation of progelatinase-A by APMA

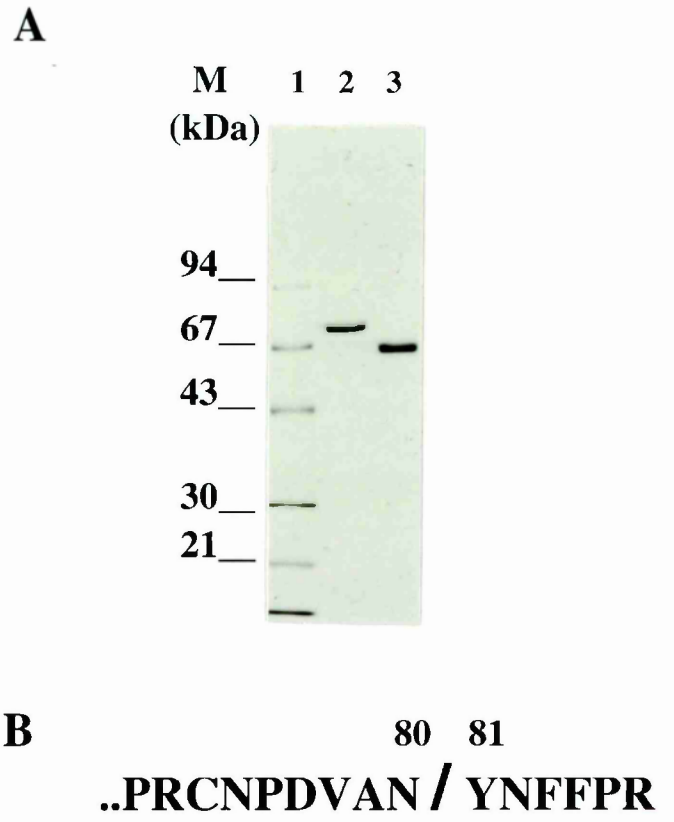
APMA induces an autocatalytic activation of progelatinase-A by a process which may be either bimolecular or unimolecular. In the following study two inhibitors TIMP-1 and a low molecular mass inhibitor,CT989, were used to study this mechanism. Both molecules are effective at comparable concentrations to those of the enzyme and this tight-binding is reflected in their low inhibition constants of $<10^{-11}$ M. The potency of these inhibitors means that they can completely block the ability of residual active enzyme (which represents 0.1% of the total enzyme in progelatinase preparations) to initiate

Figure 3.1 Time-course of progelatinase-A activation by APMA



Progelatinase-A (1 μM) was incubated at 23°C in gelatinase activation buffer and 5% DMSO in the presence of APMA (1 mM). At various times, ranging from 0 - 24 hrs, 60 μl was removed and diluted appropriately for assay against QF24 as described in Chapter 2.

Figure 3.2 Activation of progelatinase-A by APMA



(A) Progelatinase-A (2μM) was incubated with APMA (1mM) in peptide assay buffer containing 5% dimethyl sulphoxide (DMSO) for 16 hrs at 23°C. The resulting enzyme was analysed by SDS-PAGE (10 - 20%) under reducing conditions.

Lane	Sample
1	**Molecular mass markers**
2	**Progelatinase-A**
3	**Progelatinase-A after APMA treatment**

(B) The band arising from treatment with APMA (lane 3) was electroblotted onto a polyvinylidine fluoride membrane and N-terminal sequencing (Chapter 2) revealed the amino-terminus ^{81}YNFF.

the activation process. The low molecular mass inhibitor, CT989 (600 Da), may unlike TIMP, diffuse into the active-site of enzyme which has become catalytically competent through the action of APMA but has retained its propeptide. A time-course was conducted in order to test the effects of these inhibitors on the activation of progelatinase-A by APMA.

Progelatinase-A (1μM) was incubated for 10 minutes with or without TIMP-1 (4 μM) or CT989 (4 μM) prior to the addition of APMA (1mM) and 5 % DMSO. At various times ranging from 0 - 24 hrs samples were analysed by gel electrophoresis (Fig. 3.3). In the absence of inhibitor, activation correlates with the disappearance of the 72 kDa proenzyme species and the concomitant increase in the level of a 66 kDa species (Fig.3.3 {A}). The reaction proceeds *via* a major intermediate of about 67 kDa which disappears after 4 hrs incubation. Complete conversion takes place after 8 hrs and this correlates with maximal activity against QF24 (see above {i}). Addition of TIMP-1 significantly reduces the rate of formation of both the 67 kDa intermediate and the 66 kDa fully active form (Fig. 3.3 {B}). Also the conversion of the 67 kDa species to the 66 kDa form appears to be much reduced or blocked totally since its levels increase at a similar rate to the active form. Similarly, CT989 significantly reduces the rate at which the 72 kDa band is removed (Fig.3.3 {C}) but it is slightly less effective in inhibiting the production of the 66 kDa form. About 50 % of the proenzyme is converted to the lower molecular mass form after 24 hrs incubation in the presence of either inhibitor. The data indicate a process which does not involve pre-activated enzyme as this would be bound up by inhibitor. APMA may, by breaking the active-site zinc - cysteine bond, induce the enzyme to hydrolyse a bond in the vicinity of its own active site in a unimolecular process.

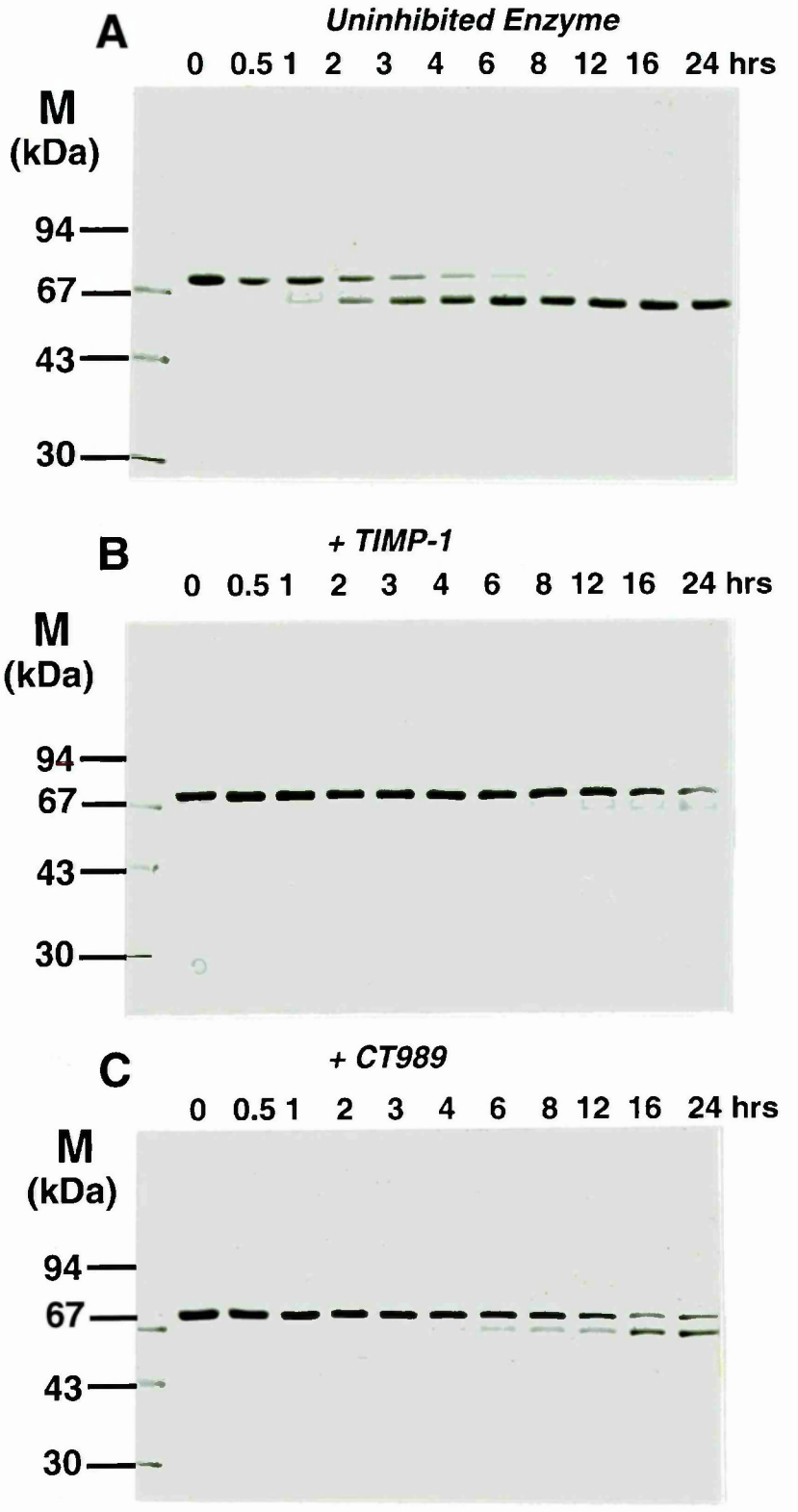
(iii) Activation of gelatinase-A by APMA : Effect of heparin

The concentration dependence of a bimolecular reaction can be used to differentiate it from a unimolecular process. Progelatinase-A can be effectively concentrated by the addition of heparin (Crabbe *et al.*, 1993). This avoids the requirement for extensive dilution which can affect the accuracy of such a study.

Figure 3.3 Inhibition of APMA-induced progelatinase-A activation

Progelatinase-A (1μM) was incubated at 23°C in gelatinase activation buffer with or without TIMP-1 (4 μM) or CT989 (4 μM). After 10 minutes a solution of APMA in DMSO was added to give a final APMA concentration of 1 mM together with 5% DMSO. At various times ranging from 0 - 24 hours, 60 μl was removed for SDS-PAGE (A - C). Samples were analysed under reducing conditions on 10 - 20% gradient gels. (A) represents uninhibited enzyme, (B) TIMP-1 and (C) CT989. Incubation times are shown above each gel and the molecular masses (M) of standard proteins are indicated. The corresponding gelatinase activity of uninhibited samples (A) is plotted in Figure 3.2.

Figure 3.3 Inhibition of APMA induced progelatinase-A activation



Progelatinase-A (1 μM) was incubated with APMA (1mM) as described above in the presence or absence of heparin (50 μg/ml). At times ranging from 0 - 6hrs samples were removed for SDS-PAGE and for assay of gelatinase activity against the QF24 substrate. The maximal activity after 6 hrs represented about 80% of that achieved by incubating progelatinase-A with APMA (1mM) for 16hrs at 23°C in peptide assay buffer (Fig. 3.4). The rate of appearance of activity was slightly faster in the presence of heparin but by 6hrs there was negligible difference in the activities of the two samples. SDS-PAGE analysis of the products of the activation time-course showed the characteristic pattern of APMA activation (Fig. 3.4 {A,B}). The 66 kDa band appeared more rapidly in the presence of heparin (Fig. 3.4 {B}). Comparison of the 1hr samples emphasises this point. At 6 hrs the 72 kDa band was still evident in both samples and this correlates with the activity observed by assay. Heparin appears to have little effect on the early steps of gelatinase autoactivation which lead to the formation of intermediates but appears to increase the subsequent rate of formation of the fully active 66 kDa species. This would suggest that the initial reaction is not concentration dependent but further cleavages are a result of intermolecular processing .

(iv) Activation of gelatinase-A with human collagenase and matrilysin

Progelatinase-A does not appear to be activated by proteinases (Okada *et al.*, 1989;1990). In the following studies matrilysin and collagenase were used to test the hypothesis that progelatinase-A possesses a bait region that is a recognition sequence for MMPs.

Progelatinase-A (3 μM) was incubated in peptide assay buffer at 37°C in the presence of either active collagenase (1 μM) or matrilysin (1 μM). An additional sample of progelatinase-A without activator was also included to test the stability of the proenzyme. At various times, ranging from 0 - 24hrs, samples were removed for SDS-PAGE and for assay of gelatinase activity.

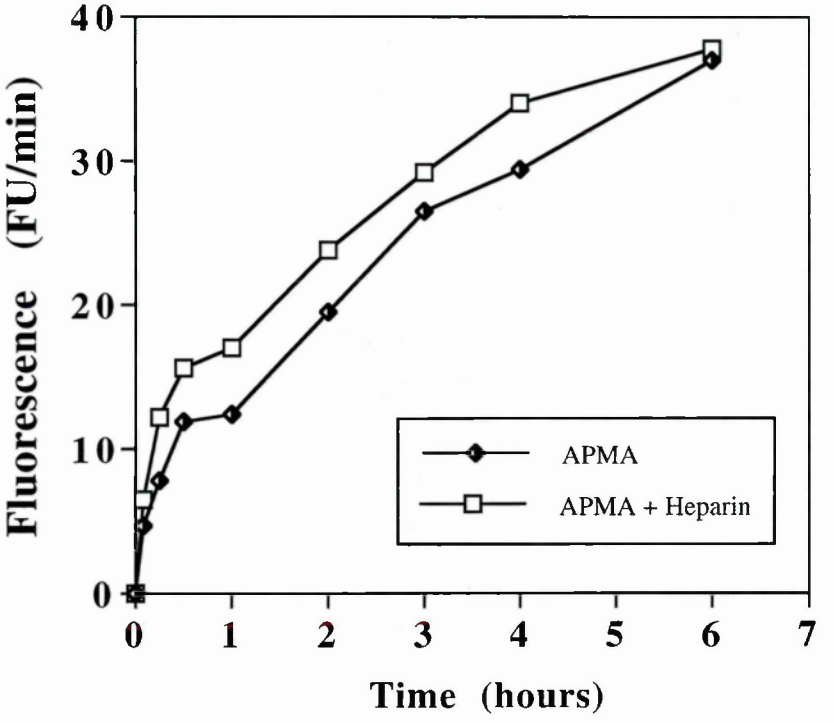
Both collagenase and matrilysin maximally activated progelatinase-A after 8hrs at 37°C (Fig. 3.5). The level of activity dropped significantly over the subsequent 16 hrs. In both cases activation correlated with the appearance of the 66 kDa band and was accompanied by a concomitant decrease in the 72 kDa proenzyme form (Fig. 3.5 (A,B)). After 16 hrs the 66 kDa species was

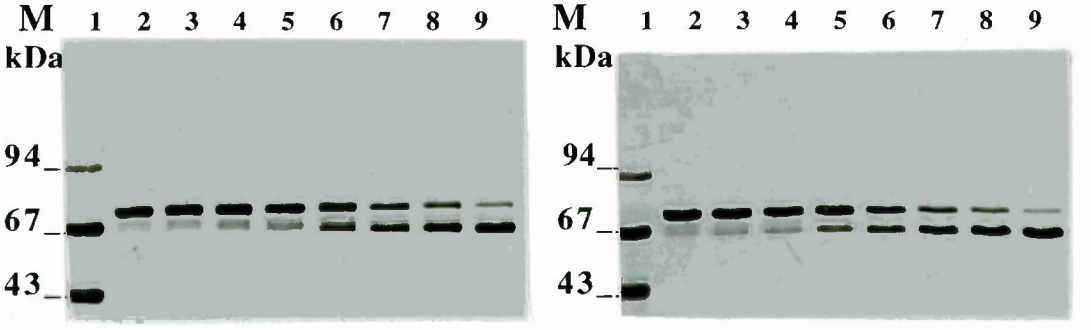
Figure 3.4 Effect of heparin on the activation of progelatinase-A by APMA

Progelatinase-A (1μM) was incubated with APMA (1mM) at 23°C in gelatinase activation buffer containing 5% DMSO in the presence or absence of heparin (50 μg/ml). At times ranging from 0 - 6 hours, 50 μl aliquots were removed for assay of gelatinase activity against QF24 and for analysis by SDS-PAGE (10 - 20%) under reducing conditions.

Lane	Sample
1	Molecular mass markers
2	5 mins
3	15 mins
4	30 mins
5	1 hour
6	2 hours
7	3 hours
8	4 hours
9	6 hours

Figure 3.4 Effect of heparin on the activation of progelatinase-A by APMA





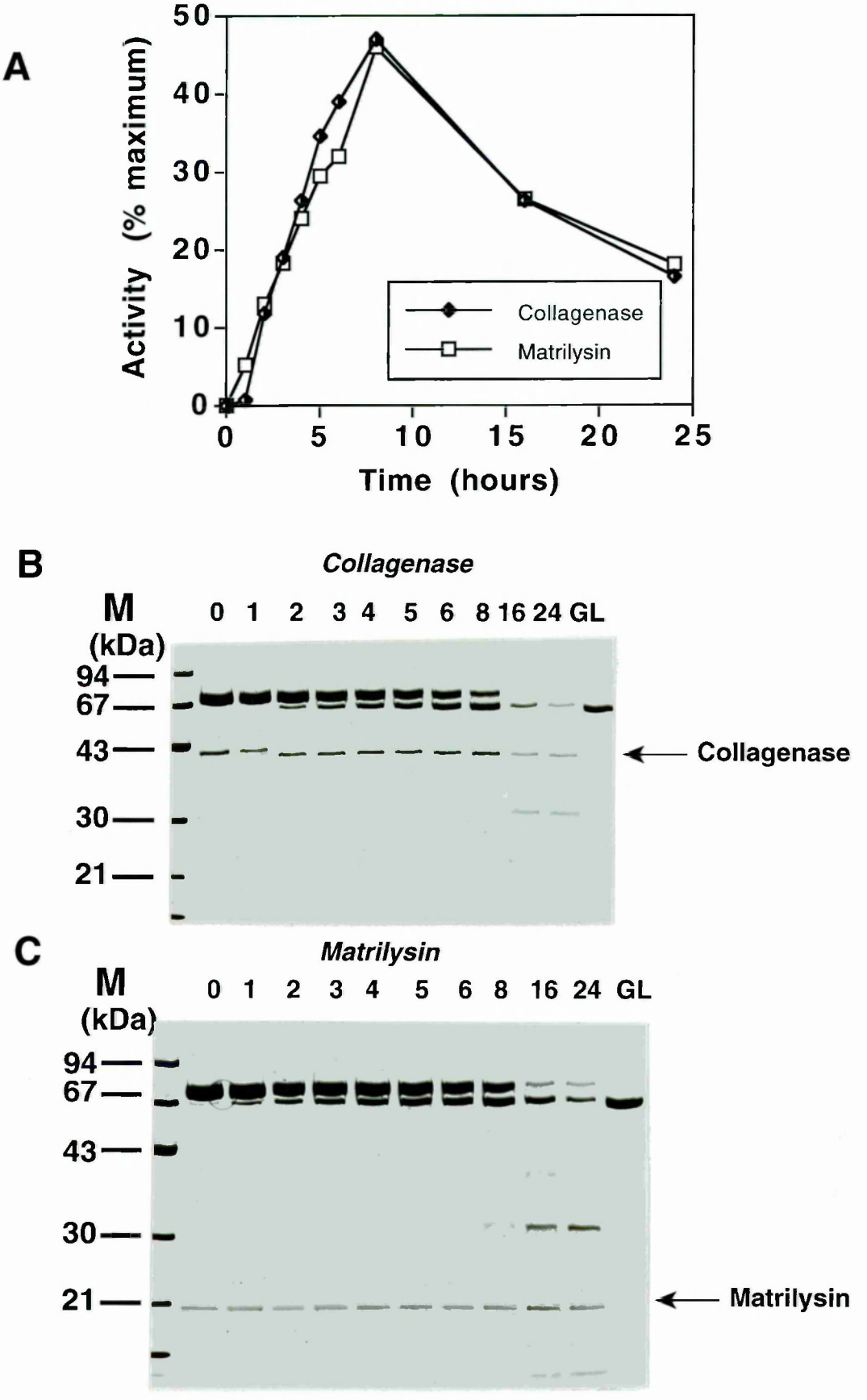
(-) Heparin (+) Heparin

Figure 3.5 Activation of progelatinase-A by collagenase and matrilysin

Progelatinase-A (3 μM) was incubated in peptide assay buffer at 37°C in the presence of either trypsin activated collagenase (1μM) or matrilysin (1μM). At various times ranging from 0 - 24 hours, 60 μl aliquots were removed for assay of gelatinase activity against QF24 (A) and for analysis by SDS-PAGE (B, C). The activity assay was performed essentially as described in Chapter 2 using an enzyme concentration equivalent to 0.3nM of the unactivated proenzyme and a substrate concentration of 1.4 μM.

SDS-PAGE analysis of the corresponding samples was carried out using 10 - 20% gradient polyacrylamide gels and (B) represents progelatinase incubated in the presence of collagenase and (C) in the presence of matrilysin. Samples were run under reducing conditions. The molecular masses (M) of standard proteins are indicated. Incubation times (hrs) are shown above each gel. APMA-activated gelatinase-A (GL) is shown for comparison.

Figure 3.5 Activation of progelatinase-A by collagenase and matrilysin



significantly lower in intensity than at the 8 hr time-point. This was accompanied by the appearance of low molecular mass species, notably a band of 32 kDa. The band corresponding to matrilysin was unaffected by the reaction. The apparent molecular mass of collagenase was reduced from 43 kDa to 42 kDa, an observation which will be discussed in Chapter 4. Progelatinase-A incubated without activator showed negligible activity after 24 hrs implying that the proenzyme is stable in peptide assay buffer for prolonged periods at 37°C.

Both collagenase and matrilysin activate progelatinase-A at 37°C to an active species which is similar in size to that generated by APMA. Unlike the proenzyme, once activated, gelatinase-A is unstable at this temperature leading to reduced activity after 16 hrs.

(v) Effect of heparin and type 1 collagen on the activation of progelatinase-A by collagenase and matrilysin

Heparin can significantly increase the rate of progelatinase-A autoactivation under low salt conditions and in the absence of Brij 35 (Crabbe *et al.*, 1993). This presumably occurs by a process in which latent and residual active enzyme are concentrated on the charged surface of the proteoglycan. Collagenase can also bind to heparin and so may substitute for the active gelatinase-A. These enzymes also bind to type 1 collagen and indeed, Azzam and Thompson (1992) recently described the ability of this agent to induce gelatinase-A activity in human cells. Concentration of the two enzymes on the surface of either of these molecules may result in an increased activation rate. The following experiments, designed to explore this possibility were carried out in gelatinase activation buffer (low ionic strength, low Brij 35) in preference to peptide assay buffer since both ionic strength and Brij 35 significantly interfere with heparin interactions.

Progelatinase-A (4μM) was incubated at 37°C with active collagenase (1μM) with or without type 1 collagen (rat skin) (50 μg/ml) or heparin (50 μg/ml). A similar study was carried out in the absence of collagenase. At times, 0hr and 4hr, samples were analysed as described in Figure 3.6.

Collagen had no effect on the activation of progelatinase-A by collagenase (Fig. 3.6 {A}). Heparin alone and in combination with collagen increased gelatinase-A activity in the presence of collagenase. There was no

Figure 3.6 Activation of progelatinase-A: Effect of activating agents

Progelatinase-A (4 μM) was incubated at 37°C with or without rat skin type 1 collagen (50 μg/ml) or heparin (50 μg/ml). An identical study was also conducted in the presence of active collagenase (CL) (1μM). At 0 hours and 4 hours, 100μl aliquots were removed for analysis of gelatinase activity against QF24 (A) and for SDS-PAGE (B). Activities are expressed as a percentage of the total activity of a sample activated by APMA for 16 hours at 23°C. The results represent the mean value of two readings. SDS-PAGE was carried out on 10 - 20% gradient polyacrylamide gels under reducing conditions. The molecular masses (M) of standard proteins (lane 1) are indicated.

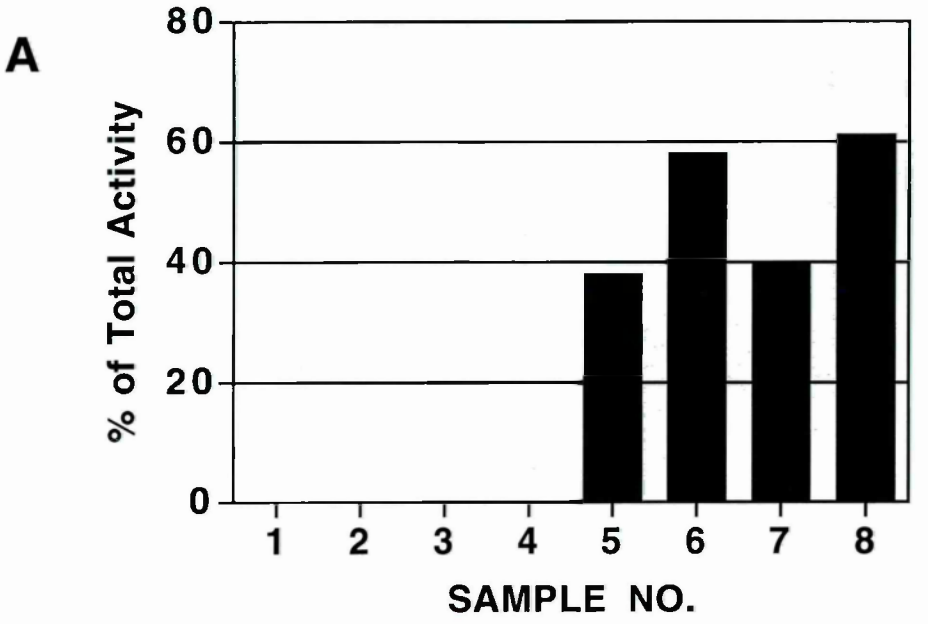
(A)

Sample no.	
1	Progelatinase-A
2	+ heparin
3	+ collagen
4	+ heparin/collagen
5	+ CL
6	+ heparin + CL
7	+ collagen + CL
8	+ heparin/collagen + CL

(B)

Lane	Sample
1	Mol. mass markers
2	Progelatinase-A (0 hrs)
3	Progelatinase-A (4 hrs)
4	+ CL (0 hrs)
5	+ CL (4 hrs)
6	+ CL + heparin (0 hrs)
7	+ CL + heparin (4 hrs)
8	+ CL + collagen (0 hrs)
9	+ CL + collagen (4 hrs)
10	+ CL + heparin/collagen (0 hrs)
11	+ CL + heparin/collagen (4 hrs)
12	Active gelatinase control

Figure 3.6 Activation of progelatinase-A: Effect of activating agents



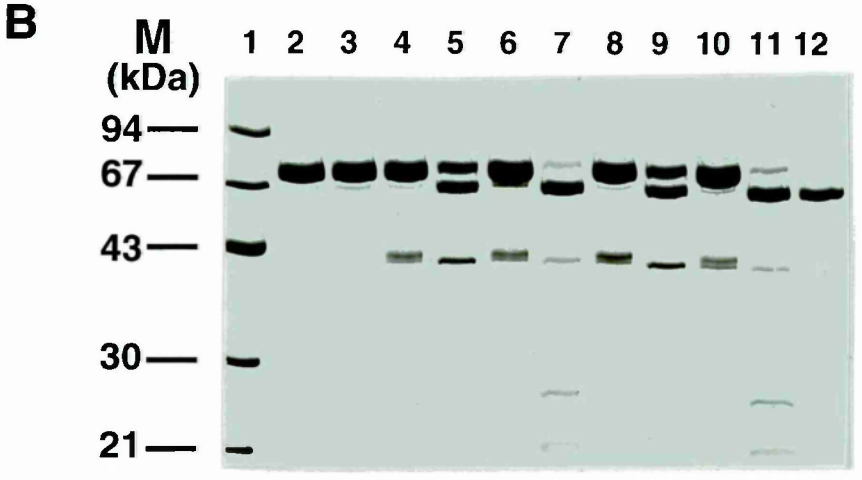
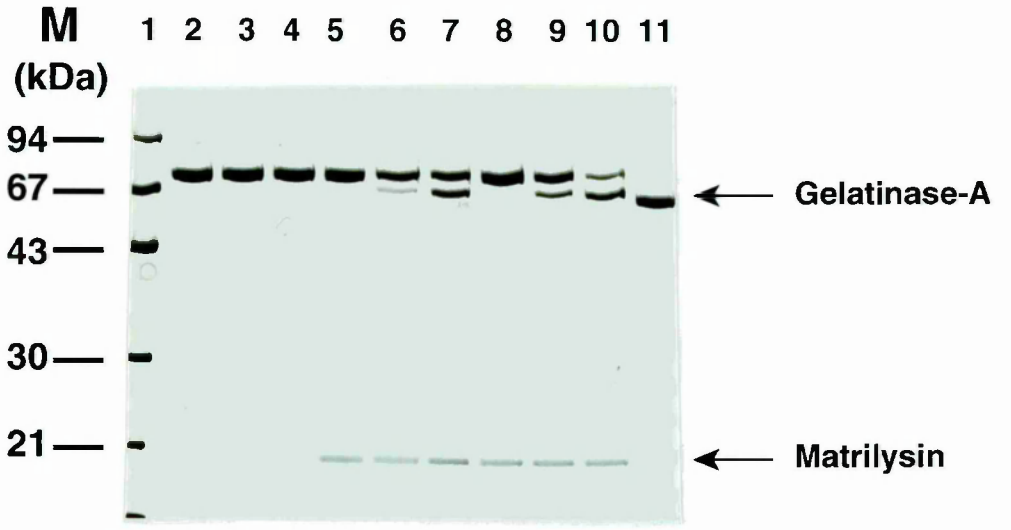


Figure 3.7 Effect of heparin on the activation of progelatinase-A by matrilysin



Progelatinase-A (1μM) was incubated with matrilysin (1μM) at 37°C in activation buffer in the presence or absence of heparin (50μg/ml). At times 0, 1 and 2 hours a 20μl aliquot was removed and analysed by SDS-PAGE (10-20%) under reducing conditions. The molecular masses (M) of standard proteins (lane 1) are indicated.

Lane	Sample	
1	Molecular mass markers	
2	0 hours	
3	1 hour	No activator
4	2 hours	
5	0 hours	
6	1 hour	Matrilysin
7	2 hours	
8	0 hours	
9	1 hour	Matrilysin/Heparin
10	2 hours	
11	APMA activated gelatinase-A control	

synergistic effect of the two reagents. No activity was observed in the absence of collagenase. SDS-PAGE of the products (Fig. 3.6 {B}) showed that the presence of collagen (lane 4,5) had little effect on the ratio of the 72 kDa proenzyme and the 66 kDa species. Heparin caused a reduction in the 72 kDa band and the production of several low molecular mass species in addition to the 66 kDa band, alone (lane 7) or in combination with collagen (lane 11).

These results indicate that heparin but not collagen stimulates the formation of active progelatinase-A by collagenase. The appearance of significant levels of low molecular mass species after 4 hrs implies that the reaction has proceeded beyond the point of maximal activity. Under these conditions heparin does not induce the activation of progelatinase-A in the absence of active enzyme.

In further experiments, heparin was tested for its effect on the activation of progelatinase-A by matrilysin. SDS-PAGE of the reaction products (Fig. 3.7) revealed that heparin does stimulate the formation of active gelatinase-A by matrilysin (lanes 8 -10). The level of progelatinase-A remaining after 2 hrs incubation with matrilysin and heparin, indicates that heparin is a less effective stimulator of matrilysin than it is of collagenase.

(vi) Time-course of heparin / collagenase activation of progelatinase-A and a truncated form, proNGL-A

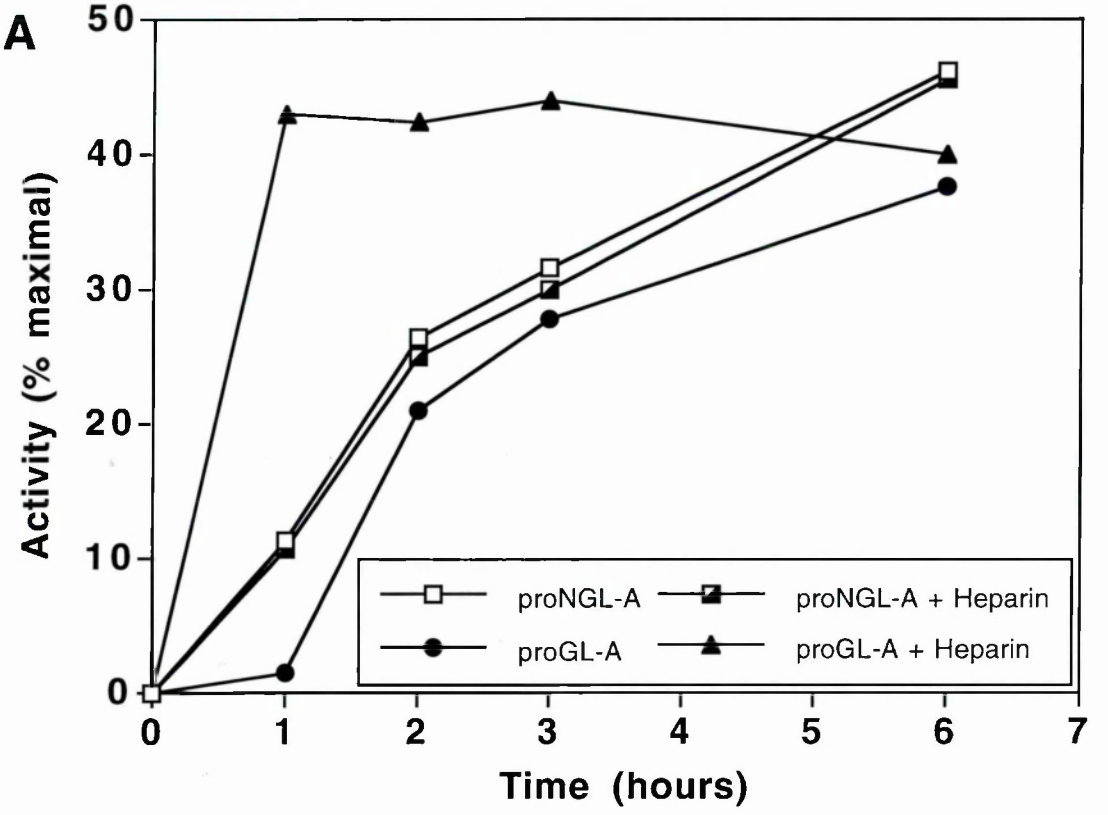
In section 3.2:1 (v) heparin was shown to increase the rate at which progelatinase-A is activated by collagenase. This was a qualitative study that was carried out under sub-optimal conditions. The following study examines the rate of progelatinase-A activation by an equimolar level of collagenase with and without heparin. Additionally, the importance of the gelatinase C-terminal domain to heparin activation was studied by conducting a parallel experiment using a truncated gelatinase which lacked this domain, progelatinase $_{1-417}$ (proNGL-A)).

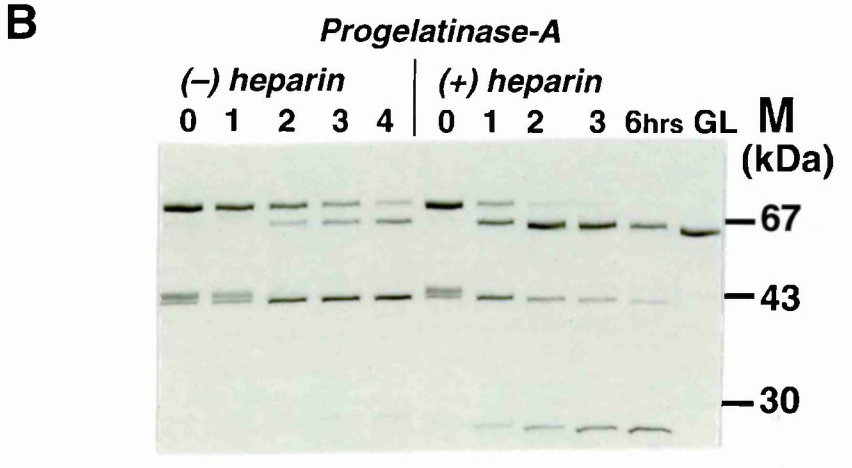
Progelatinase-A (1 μM) or proNGL-A (1μM) were incubated with active collagenase with or without heparin (50 μg/ml). At various times ranging from 0 - 6hrs, samples were removed for SDS-PAGE and assay of gelatinase activity. In the absence of heparin the activity of progelatinase-A (expressed as a percentage of that achieved by APMA) increased with time to reach maximal activity at 6 hrs (Fig. 3.8 {A}). Addition of heparin significantly increased the

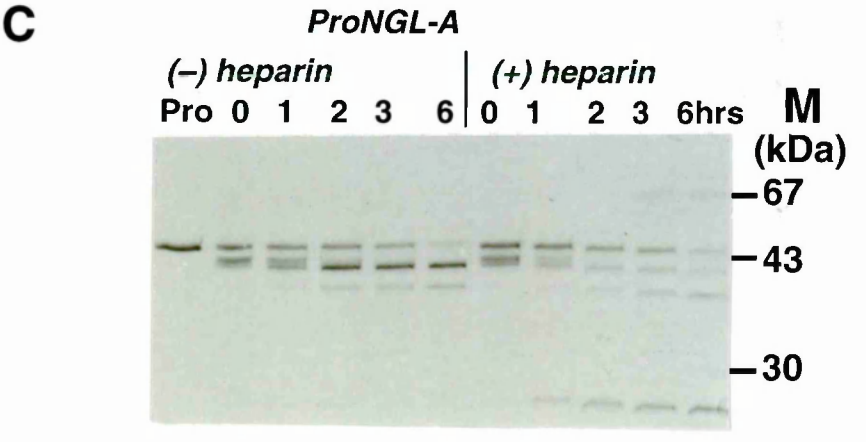
Figure 3.8 Activation of progelatinase-A and proNGL-A by collagenase and heparin

Progelatinase-A or proNGL-A (1 μM) were incubated in gelatinase activation buffer at 37°C with active collagenase (1μM) in the presence or absence of heparin (50 μg/ml). At times 0 , 1, 2, 3 and 6 hrs, 50μl aliquots were removed for assay of gelatinase activity (A) using QF24 and for SDS-PAGE (B,C). Activities are expressed as a percentage of the total activity of a sample activated by APMA for 16 hours at 23°C. The results represent the mean of two readings. SDS-PAGE was carried out on 10 - 20% polyacrylamide gradient gels under reducing conditions. Incubation times (hrs) and the presence or absence of heparin are indicated above each gel. Controls include GL (gel B) which is APMA-activated gelatinase-A and Pro (gel C) which is unactivated proNGL-A. The molecular masses (M) of protein standards are indicated.

Figure 3.8 Activation of progelatinase-A and proNGL-A by collagenase and heparin







rate of progelatinase-A activation and resulted in maximal activity after 1 hr. Activity did not increase further over the subsequent 5 hrs. ProNGL-A was activated by collagenase at a rate slightly faster than the parent form but its rate of activation was not affected by heparin. SDS-PAGE analysis of the products of progelatinase-A activation (Fig. 3.8 {B}) showed that activity correlated with the appearance of a species of 66 kDa. In the presence of heparin this appeared rapidly before decreasing with the subsequent generation of low molecular mass species (lanes 7-11).

SDS-PAGE of the products arising from proNGL-A activation indicated that the generation of activity was associated with a reduction in molecular mass from 45 kDa to 37 kDa (Fig. 3.8 {C}). Addition of heparin (lanes 7-11) had no observable effect on the generation of the 37 kDa species. In summary, heparin significantly increases the rate of progelatinase-A activation by collagenase in a process which involves the C-terminal domain.

(vii) Effect of heparin concentration on the activation of progelatinase-A and proNGL-A

The studies of Crabbe *et al.* (1993) showed that the rate of autoactivation of progelatinase-A was dependent on the heparin concentration and that excessive heparin can be detrimental to the reaction. This implies that heparin works by localising the enzymes on its surface, a process which would be reversed if it were in excess. The following study was conducted to test the effect of varying the heparin concentration on collagenase activation of progelatinase-A and whether or not the response of proNGL-A to heparin is concentration dependent.

The two proenzymes were incubated with collagenase as described in section 3.2:1 (v), together with heparin at concentrations ranging from 0 - 1mg/ml. After 40 minutes at 37°C, 50 µl aliquots were removed for SDS-PAGE and gelatinase assay. The rate at which progelatinase-A was activated by collagenase increased rapidly with increasing heparin concentration (Fig. 3.9 {A}). Maximal activity was observed at a concentration of 200 µg/ml and this activity remained relatively constant up to a heparin concentration of 1mg/ml. Heparin had no effect on the activation of proNGL-A by collagenase. Furthermore, neither progelatinase-A nor proNGL-A responded to heparin in the absence of collagenase.

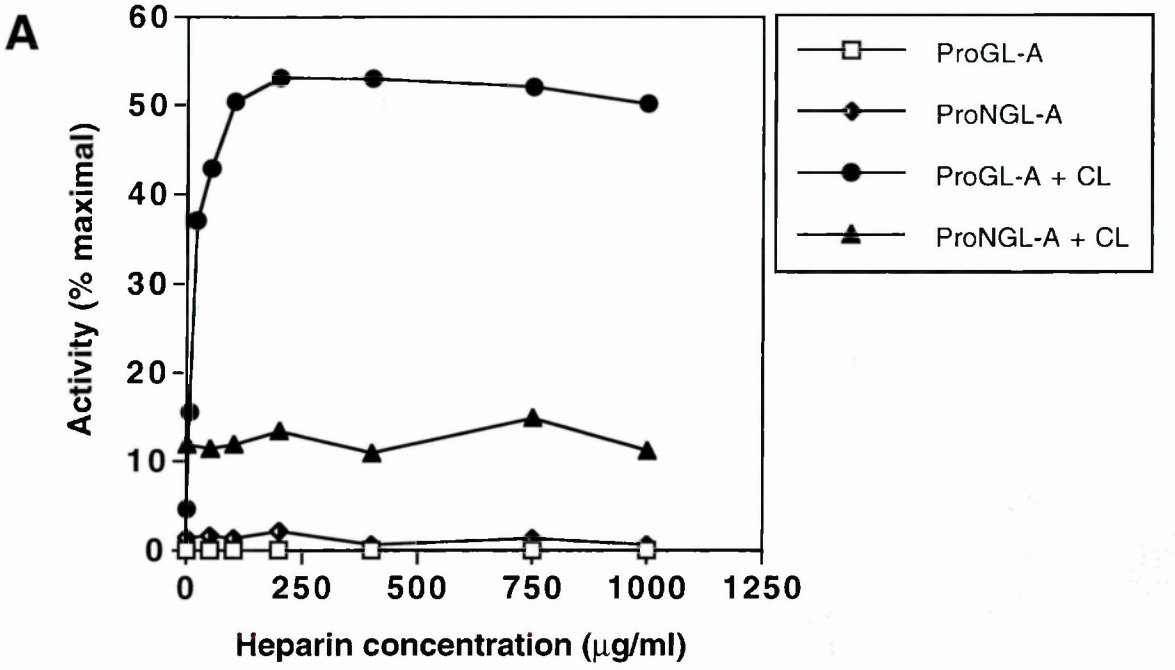
Figure 3.9 Effect of heparin concentration on collagenase activation of progelatinase-A and proNGL-A

Progelatinase-A (proGL-A) or proNGL-A (1μM) were incubated in gelatinase activation buffer at 37°C with or without active collagenase (CL) (1μM) in the presence of heparin at concentrations ranging from 0 - 1mg/ml. After 40 minutes, 50μl aliquots were removed for assay of gelatinase activity (A) using QF24 and representative samples were analysed using SDS-PAGE (B). Activities are expressed as a percentage of the total activity of a sample activated by APMA for 16 hours at 23°C. The results represent the mean of two readings. SDS-PAGE was carried out on a 4 - 20% polyacrylamide gradient gel under reducing conditions.

(B)

Lane	Sample
1	**Molecular mass markers**
2	**ProGL-A + CL**
3	**ProGL-A + CL + heparin (10 μg/ml)**
4	**ProGL-A + CL + heparin (100 μg/ml)**
5	**ProGL-A + CL + heparin (1mg/ml)**
6	**ProGL-A control**
7	**ProNGL-A + CL**
8	**ProNGL-A + CL + heparin (10 μg/ml)**
9	**ProNGL-A + CL + heparin (100 μg/ml)**
10	**ProNGL-A + CL + heparin (1mg/ml)**
11	**ProNGL-A control**

Figure 3.9 Effect of heparin concentration on collagenase activation of progelatinase-A and proNGL-A



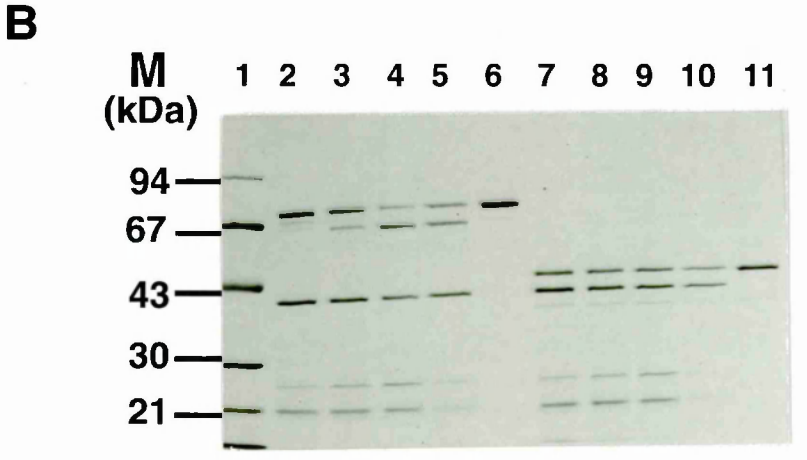
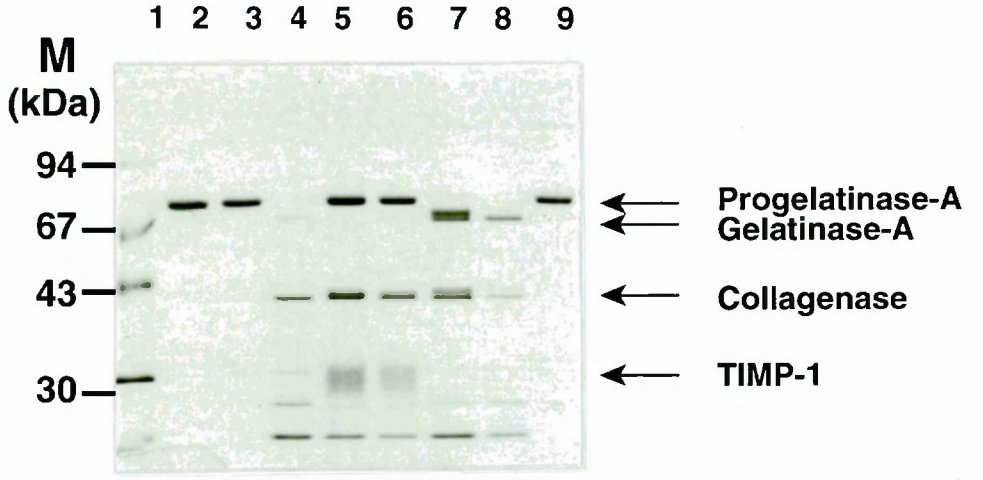


Figure 3.10 Effect of inhibitors on the activation of progelatinase-A by collagenase



Progelatinase-A (1μM) samples were incubated with collagenase (CL) (1μM) and inhibitor (4μM) as described in the text (section 3.2 (viii)). Aliquots were analysed by SDS-PAGE (4-20%) under reducing conditions.

Lane	Sample
1	Molecular mass markers
2	Progelatinase-A (0 hours)
3	Progelatinase-A (24 hours)
4	Progelatinase-A + CL (24 hours)
5	Progelatinase-A + CL + TIMP-1 (24 hours)
6	Progelatinase-A + CL + TIMP-1 (48 hours)
7	Progelatinase-A + CL + CT989 (24 hours)
8	Progelatinase-A + CL + CT989 (48 hours)
9	Progelatinase-A (0 hours)

SDS-PAGE was carried out on representative samples (Fig. 3.9 {B}). Generation of the 66 kDa band correlates with the activity of the full-length enzyme. It is evident, however, that there is less conversion of the 72 kDa proenzyme at a heparin concentration of 1mg/ml (lane 5) than at 0.1mg/ml (lane 4) and that this does not correlate with the corresponding levels of activity in the assay. This apparent discrepancy can be rationalised by observation of the relative levels of low molecular mass breakdown products (27 kDa) in the two samples. These bands are increased in the 0.1mg/ml sample although the active 66 kDa band remains constant. Excess heparin can, therefore, inhibit the conversion of pro- to active gelatinase-A by collagenase. Gel electrophoresis of the proNGL-A activation by collagenase (lanes 7-11) confirmed the assay results showing that heparin had no effect at concentrations up to 1mg/ml.

(viii) Effect of inhibitors on collagenase activation of gelatinase-A

TIMP-1 inhibits collagenase and gelatinase-A with a Ki_{app} of $<10^{-10}$ M and 10^{-12} M respectively. The synthetic hydroxamate inhibitor, CT989, (Chapter 5) is more specific for gelatinase than collagenase (Ki_{app} is $<10^{-11}$ M and 3.29×10^{-7} M, respectively). By using inhibitors of differing specificity the relative contributions of gelatinase and collagenase to the activation process can be estimated.

Progelatinase-A (1 μM) was incubated in gelatinase activation buffer with collagenase (1μM) at 37°C. Additional samples were incubated as described above but in the presence of TIMP-1 (4 μM) or CT989 at two concentrations (0.1μM and 1μM). After 24hrs and 48hrs, 100μl aliquots were removed. Samples were analysed by SDS-PAGE under reducing conditions (Fig. 3.10). TIMP-1 completely blocked the activation of progelatinase-A by collagenase at 24hrs and 48hrs (lanes 5 and 6 respectively). At a concentration of 0.1μM, CT989 had little effect on the processing of the 72 kDa form (data not shown) but at 1μM it did reduce the rate of conversion seen after 24 hrs (lane 7). At this point an intermediate can be observed in addition to the 66 kDa active species. Complete hydrolysis of the 72 kDa band was, however, evident after 48 hrs incubation at 37°C (lane 8). CT989 at concentrations up to 1μM does not significantly inhibit the hydrolysis of 72 kDa gelatinase by collagenase but it does block the subsequent step which generates the 66 kDa

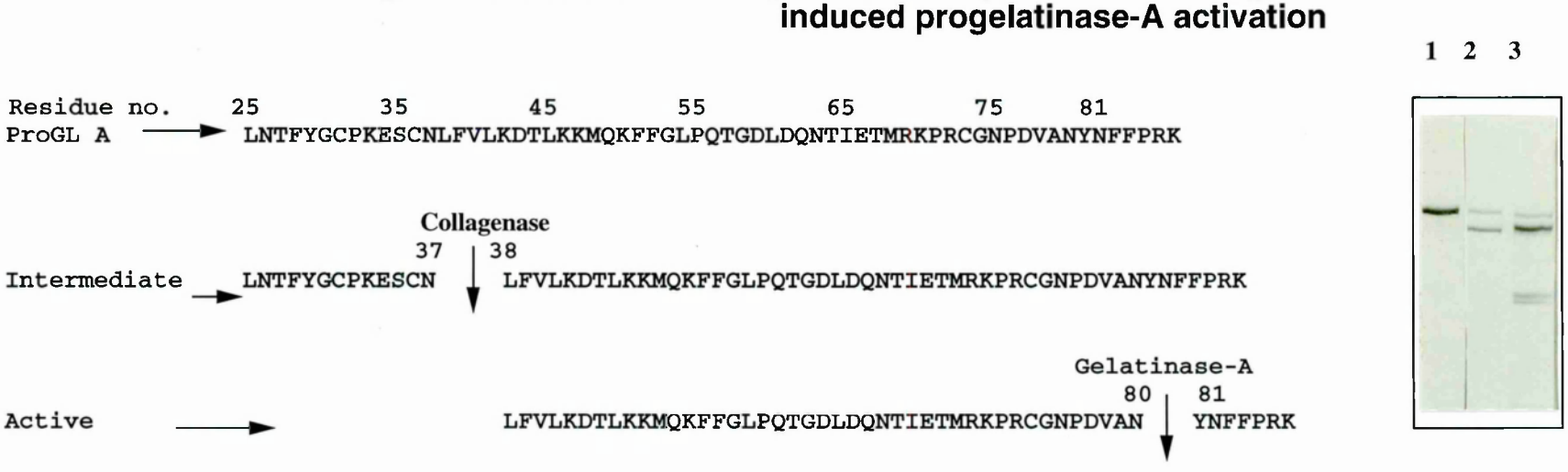
enzyme form. The results indicate that collagenase is responsible for the initial cleavage of progelatinase-A resulting in the generation of an intermediate form. This step is not inhibited by the relatively poor collagenase inhibitor CT989 but is inhibited by TIMP-1. The conversion of the intermediate to the 66 kDa active form is a product of self-cleavage. These results are consistent with the findings of Dr. T. Crabbe (personal communication) who showed that incubation of collagenase with catalytically inactive progelatinase-A ($E^{375}\rightarrow A$) results in the production of an intermediate which is not processed further.

(ix) Sequence of the activation intermediate produced by collagenase

Activation of progelatinase-A by TPA stimulated HT1080 cells proceeds *via* an intermediate with the N-terminal residue L^{38} (Strongin *et al.*, 1993). The studies above (section viii) suggest that the synthetic inhibitor CT989, may be used to isolate an intermediate produced by the action of collagenase on progelatinase-A. The N-terminal sequence of this intermediate may then be established by analysis of the electroblotted band.

Progelatinase-A (3 μM) was incubated with collagenase (1μM) and heparin (50 μg/ml) in gelatinase activation buffer for 6hrs at 37°C in the presence or absence of CT989 (3 μM). Samples were run on an SDS-polyacrylamide gel and then bands representing the intermediate (68 kDa) and the fully processed gelatinase-A (66 kDa) were then N-terminally sequenced following electroblotting as described in Chapter 2. Sequence analysis (Fig. 3.11) indicates that in the absence of CT989 incubation of progelatinase-A with collagenase produces an N-terminus Y^{81} which is identical to that generated by APMA. Activation of progelatinase-A by collagenase, in the presence of CT989 to inhibit gelatinase activity, resulted in the generation of an intermediate formed by the cleavage of the bond N^{37} - L^{38}. This latter observation is consistent with the possibility that collagenase initiates the membrane mediated activation of progelatinase-A by TPA stimulated HT1080 cells.

Figure 3.11 N-terminal sequence of the products of collagenase - induced progelatinase-A activation



A

Progelatinase-A (3μM) was incubated with trypsin activated collagenase (1μM) in the presence or absence of a specific inhibitor, CT989.

Samples were electrophoresed on a 10 - 20 % gradient polyacrylamide gel and then sequenced after electroblotting onto a polyvinylidine fluoride membrane (Chapter 2). The points of cleavage are shown above. The corresponding polyacrylamide gel is shown in (A). Lanes represent (1) 72 kDa progelatinase, (2) active 66kDa gelatinase (lower band) and (3) 68kDa gelatinase intermediate.

3.2:2 Kinetic analysis of gelatinase-A activity

N-terminal sequencing and gel electrophoresis show that a single form of gelatinase-A (Y^{81}) can be generated by the action of APMA. This permits the analysis of kinetic properties such as k_{cat}/K_m to be carried out accurately and reproducibly. The specific activity of wild-type gelatinase-A was tested on a macromolecular substrate, gelatin and on two fluorescent peptide-like substrates, DnpPLGLWAR and QF24 to determine the best substrate for inhibitor studies.

(i) Determination of a composite K_m and k_{cat}/K_m on gelatin

Labelled [^{14}C]-gelatin was prepared as described in Chapter 2. This was diluted with a range of concentrations of unlabelled gelatin and the specific activity of the label in each was calculated.

APMA activated gelatinase A (1nM) was incubated at 25°C in gelatin assay buffer with radiolabelled substrate at concentrations ranging from 0.18 μM - 2.5 μM. At times, 0 and 30 mins, 300 μl of reaction mixture was removed and processed using TCA precipitation as described previously (section 2.2:6). The hydrolysis rate at each substrate concentration was determined by converting radioactivity (cpm) in the TCA supernatant to μg of gelatin hydrolysed in unit time (min). The Enzfitter programme (Leatherbarrow) was used to calculate the K_m from a plot of rate (μg/ml) (y - axis) *versus* substrate concentration (μM) (x - axis). Values for k_{cat} and k_{cat}/K_m were determined from the relationship, $k_{cat} = V_{max}/[E]$ where V_{max} is the maximum rate of substrate hydrolysis attainable by a fixed concentration of enzyme [E] (Table 3.1).

Gelatinase-A cleaves gelatin at multiple sites. Values for k_{cat} and K_m are, therefore, not true constants but are a composite of a number of individual microscopic constants. Nonetheless, the experimentally determined k_{cat}/K_m does indicate that gelatinase-A has a high specific activity against gelatin and this is useful information for the design of gelatin based screening assays. Because gelatin is, in effect, a mixture of substrates it is unsuitable for detailed kinetic analysis.

Table 3.1

Specific activity of gelatinase-A

SUBSTRATE	kcat (s^{-1})	Km (μM)	kcat/Km ($M^{-1}s^{-1}$)
GELATIN	0.55[a]	1.24[a]	4.47×10^{5}[a]
Dnp-PLGLWAR	6.37	70	9.1×10^{4}
QF24	ND[b]	ND[b]	6.11×10^{5}

a These are not true kinetic constants (see text, section 3.2:2 (i))

b Insolubility of substrate prevents direct determination of k_{cat} and K_m

Progelatinase-A was activated with APMA (1 mM) for 7 hours at 23°C.

Dnp-PLGLWAR peptide and gelatin assays were conducted at 26°C and QF24 assays at 23°C. Values represent the mean of at least 3 readings.

Experimental procedures for determining the above kinetic constants are described in the text (3.2:2 {i - iii}).

(ii) Determination of the k_{cat}/K_m on DnpPLGLWAR

APMA activated gelatinase-A (0.1nM) was incubated in peptide assay buffer at 26°C in the presence of a variety of substrate concentrations, ranging from 8 - 100 μM. After 3 - 5 hrs assays were terminated and the rates of hydrolysis were measured as described in section 2.2:2. Endpoints were varied according to the substrate concentration such that the reaction was linear with time and < 5% of the substrate was hydrolysed. The Enzfitter programme was then used to generate k_{cat} and k_{cat}/K_m (Table 3.1).

(iii) Determination of the k_{cat}/K_m on QF24

A K_m value for QF24 was not determined due to the lack of substrate solubility, in addition to the effect of fluorescence quenching at high substrate concentrations. The evidence of limited studies would suggest that the value is >30μM. The k_{cat}/K_m was, therefore, determined directly by using a substrate concentration of 1.4 μM which satisfies the condition $S << K_m$ (see below).

Assays were performed at 23°C in peptide assay buffer at an enzyme concentration of 0.05 - 0.1nM. The rate of substrate hydrolysis was measured continuously for 1 - 2 mins over which time there was no indication of substrate depletion or quenching by product. The k_{cat}/K_m (Table 3.1) was determined using the relationship,

$$\frac{k_{cat}}{K_m + s} = \frac{Rate}{s.[E]} M^{-1}s^{-1}$$

where the $K_m + S$ term approaches K_m at values of $S << K_m$.

Gelatinase-A has a high specific activity against gelatin and the two peptides, DnpPLGLWAR and QF24. The k_{cat}/K_m values against the peptides compare favourably with those of collagenase, matrilysin and stromelysin (Chapter 5 and data not shown). Its high specific activity against these substrates permits the analysis of very low concentrations of enzyme. In fact, 20 pM enzyme hydrolyses QF24 at a rate which can be measured with sufficient accuracy to calculate inhibitor Ki's as low as 10 pM. The significance of this finding will be discussed in later chapters.

3.2:3 Progelatinase-B activation

The activation of progelatinase-B has been studied by a number of groups, but the data emerging are not consistent. Progelatinase-B may be activated by trypsin and APMA and can also be directly activated by stromelysin. The studies here were undertaken in order to address the following issues;

1. The comparative activation rates of several different activating agents
2. The specific activity of the resulting active species
3. Characterisation of the active species by N-terminal sequencing
4. The role of the C-terminal domain during activation

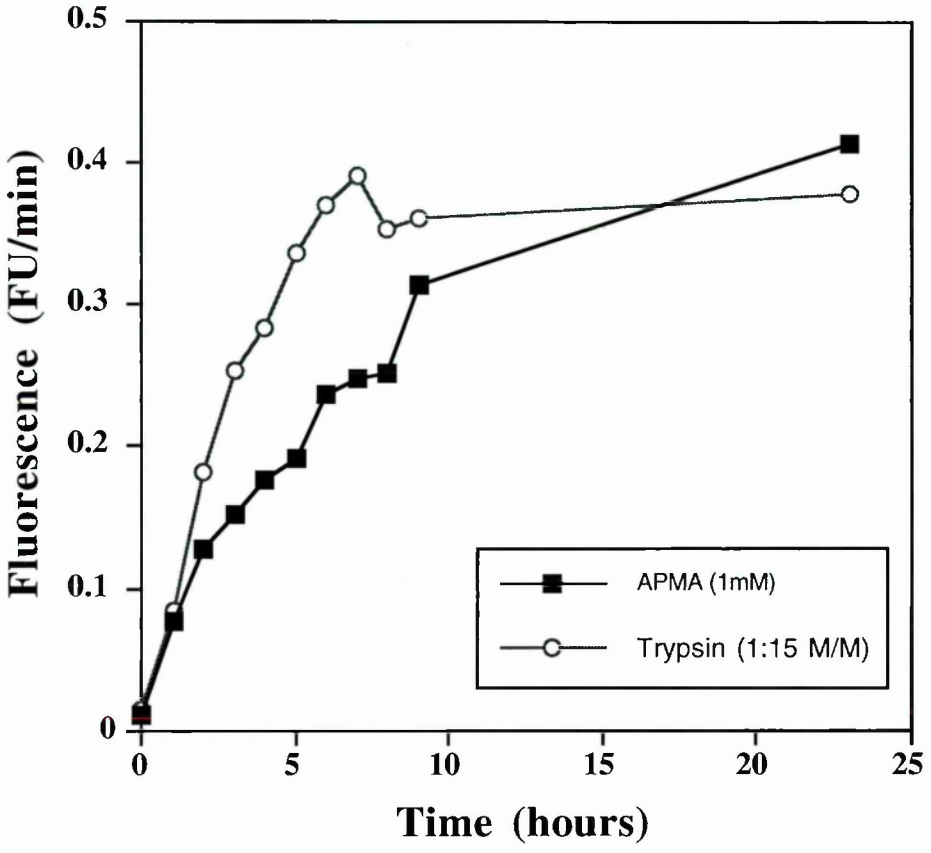
(i) Comparison of trypsin activation to APMA activation

Progelatinase-B was incubated at 37°C in peptide assay buffer in the presence of trypsin (0.13μM) or APMA (2mM), the latter contributing 10% DMSO to the reaction mixture. At various times, ranging from 0 - 22 hrs, 20μl was removed for SDS-PAGE together with 10μl for the assay of gelatinase activity. The rate of gelatinase activation was followed using DnpPLGLWAR.

Optimum activity was achieved after 7 hrs incubation with trypsin (Fig. 3.12). This was associated with the production of an 82 kDa species on SDS-PAGE under non-reducing conditions (Fig. 3.13 {B}) which decreased to 70 kDa following reduction (Fig. 3.13 {A}). Activation using APMA was somewhat slower, taking 22 hrs to reach maximum activity (Fig. 3.12). SDS-PAGE of the reaction products revealed a time-dependent increase of a major species running at 66 kDa under reducing conditions (Fig. 3.13 {D}). Analysis of these samples using non-reducing conditions showed that the appearance of a band of 68 kDa correlated with enzyme activity (Fig. 3.13 {C}).

Both these methods caused additional hydrolysis at sites within the C-terminal domain as shown by gel electrophoresis using reducing conditions. Gel electrophoresis of APMA treated samples using non-reducing conditions revealed the production with time of a 68 kDa species which indicates that the entire C-terminal domain is eventually removed.

Figure 3.12 Activation of progelatinase-B by trypsin and APMA

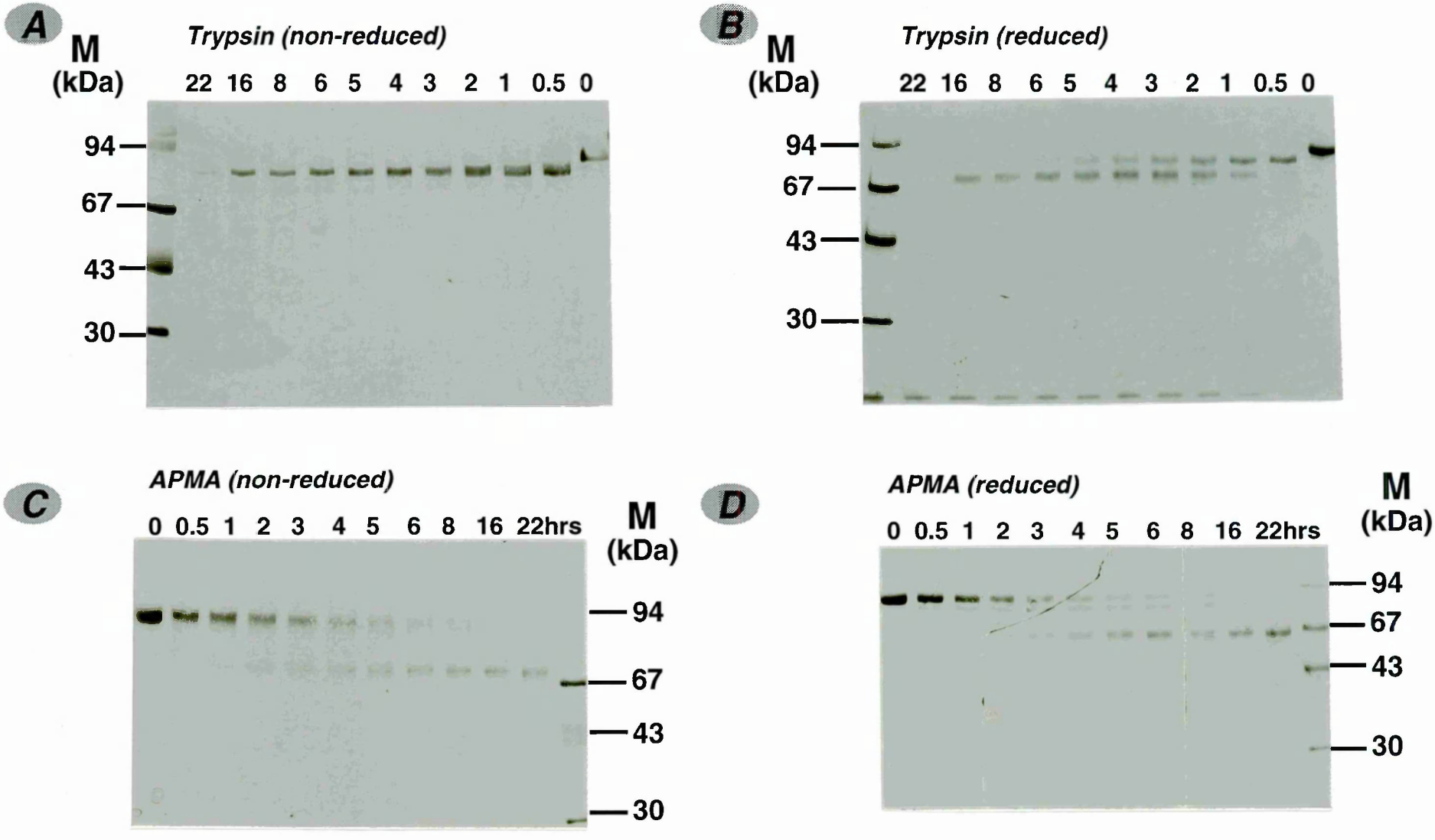


Progelatinase-B (2 μM) was activated using either trypsin or APMA as described in the text (3.2:3 {i}). The rate of activation was followed by measuring the activity of appropriately diluted samples against DnpPLGLWAR. Corresponding samples were also analysed by SDS-PAGE (Fig. 3.13, A - D).

Figure 3.13 Analysis of progelatinase-B activation by trypsin and APMA using SDS-PAGE

Progelatinase-B (2 μM) was activated using either trypsin or APMA as described in the text (3.2:3 {i}). At times ranging from 0 - 22 hours, aliquots were removed for analysis of gelatinase activity (see Fig. 3.12) and for SDS-PAGE (A - D). Samples were electrophoresed on 10 - 20% gradient polyacrylamide gels under both non-reducing (A,C) and reducing conditions (B,D). Trypsin-activated samples are analysed on gels (A) and (B) and APMA samples on gels (C) and (D). The molecular masses (M) of standard proteins are indicated. Incubation times (hrs) are shown above each gel.

Figure 3.13 Analysis of progelatinase-B activation by trypsin and APMA using SDS-PAGE



(ii) Comparison of APMA and stromelysin activation of progelatinase-B and $\Delta_{426-688}$ progelatinase-B (proNGL-B) which lacks the C-terminal domain

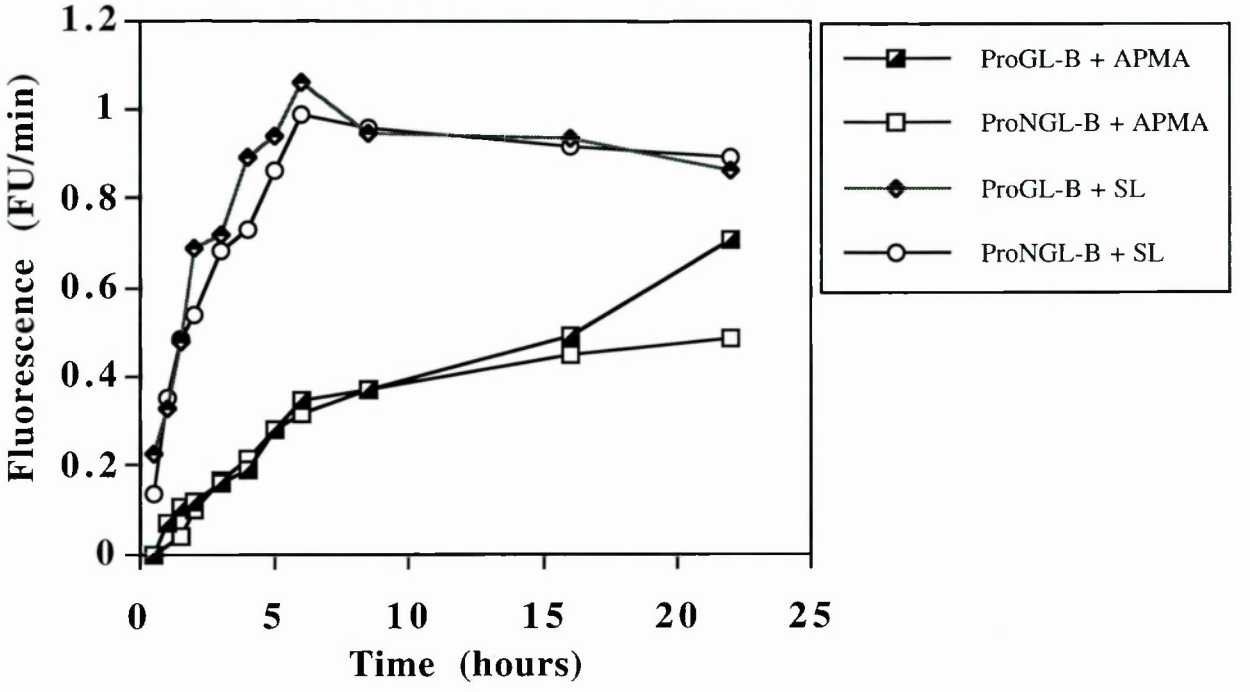
Progelatinase-B (2 μM) or proNGL-B (2 μM) were incubated with stromelysin (0.133 μM) or APMA (2 mM) under conditions essentially as outlined above (3.2:5. (i)). Samples were removed at times ranging from 0 - 22 hrs and then analysed by SDS-PAGE and by assay against DnpPLGLWAR.

Both forms of the enzyme were maximally activated after 6 hrs in the presence of stromelysin (Fig. 3.14). The products resulting from stromelysin activation of progelatinase-B gave a similar pattern when analysed by SDS-PAGE under both reducing (Fig. 3.15 {A}) and non-reducing conditions (Fig. 3.15 {B}). A species of 86 kDa was generated initially and this was subsequently converted to an 82 kDa form. Activation of proNGL-B by stromelysin correlated with a reduction in molecular mass from 55 kDa to 40 kDa via an intermediate of 42 kDa under both reducing (Fig. 3.15 {C}) and non-reducing conditions (Fig. 3.15 {D}). Activation of progelatinase-B and proNGL-B in the presence of APMA was much slower and gave maximal activity at about 22 hrs (Fig. 3.14). The active form of proGL-B had a molecular mass of 66 kDa and was formed *via* several intermediates (Fig. 3.13 {C,D}) as reported in section 3.2:3 (i). Activation of proNGL-B by APMA involved the loss of a 3 kDa fragment before the generation of a fully processed product migrating at 40 kDa under both reducing (Fig.3.15 {E}) and non-reducing conditions (Fig. 3.15 {F}).

N-terminal sequencing of the active species of the gelatinases showed that stromelysin activation generates F^{81} at the N-terminus of both the full-length and the truncated enzyme whereas APMA induces the cleavage of the bond between A^{74} and M^{75} for both enzyme forms (Table 3.2).

The C-terminal domain of progelatinase-B does not appear to influence either the APMA induced autolytic activation of the enzyme or the stromelysin catalysed removal of the propeptide region.

Figure 3.14 Activation of progelatinase-B and proNGL-B by stromelysin and APMA

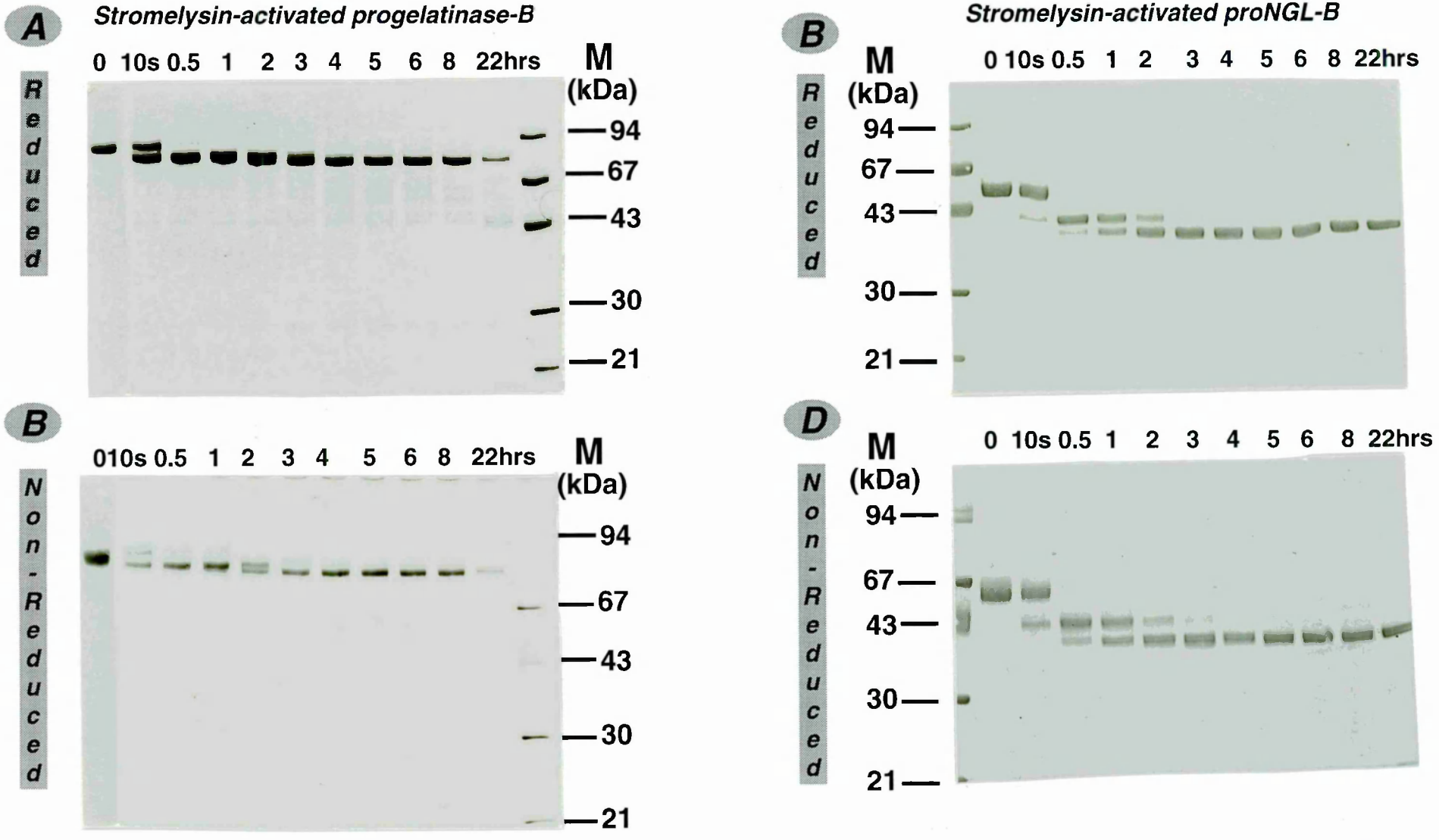


Progelatinase-B (proGL-B) or proNGL-B (2 μM) were incubated at 37°C with trypsin-activated stromelysin (SL) (0.17 μM) or APMA (2 mM) and 10% DMSO. At various times, ranging from 0 - 22 hrs an aliquot was removed for assay against DnpPLGLWAR. Corresponding samples were also analysed by SDS-PAGE under both non-reducing and reducing conditions (Fig. 3.13 {C, D}, Fig. 3.15 {A - F}).

Figure 3.15 SDS-PAGE analysis of progelatinase-B and proNGL-B activated by stromelysin and APMA

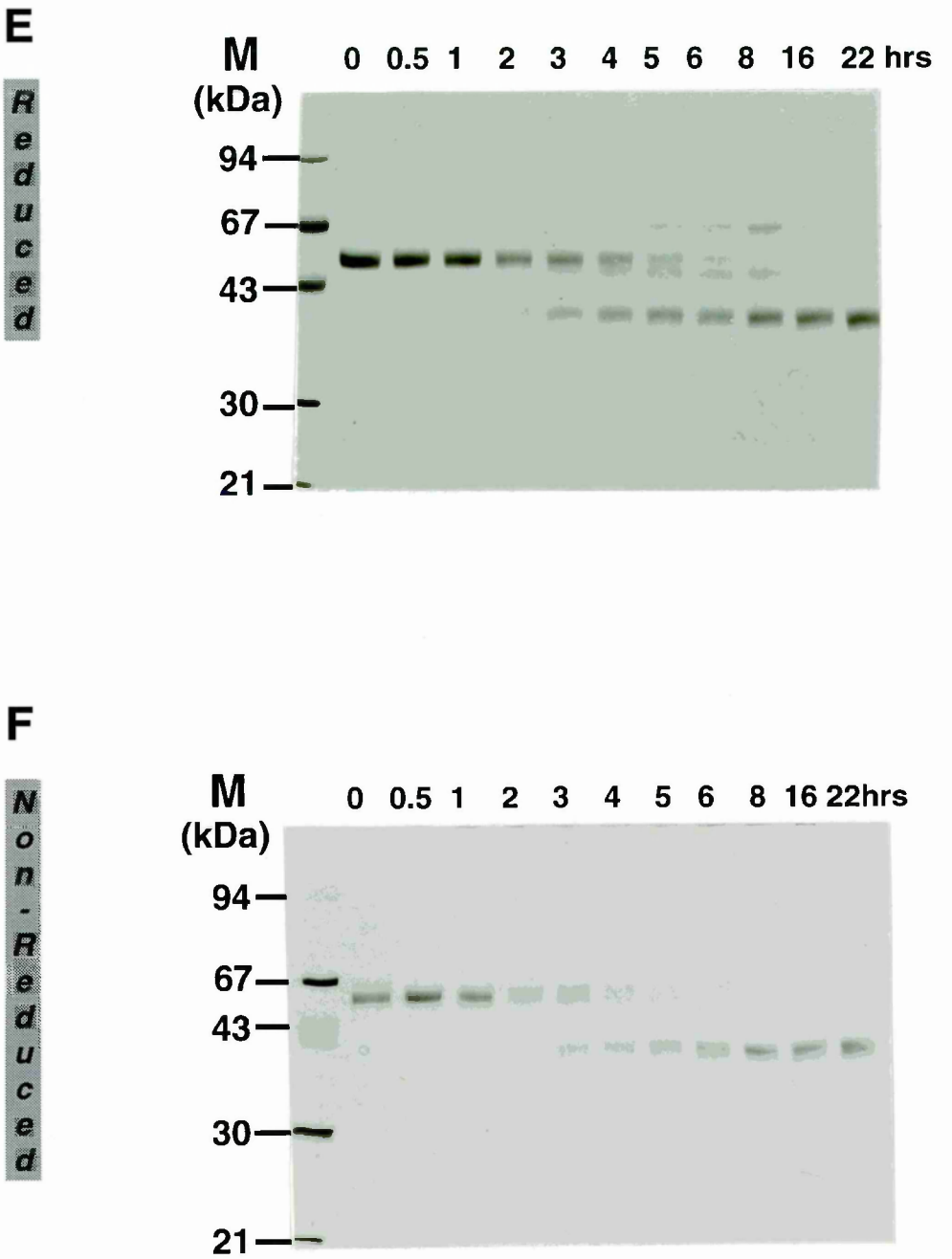
Progelatinase-B (2μM) or proNGL-B (2μM) were activated using either stromelysin or APMA as described in Figure 3.14. At times ranging from 0 - 22 hours, aliquots were removed for analysis of gelatinase activity (Fig. 3.14) and for SDS-PAGE (A - F). Samples were electrophoresed on 10 - 20% gradient polyacrylamide gels under both reducing (A,C,E) and non-reducing (B,D,F) conditions. Stromelysin activation of full-length proGL-B and proNGL-B is demonstrated on gels (A, B) and (C, D) respectively. APMA activation of proNGL-B is demonstrated on gels (E) and (F). APMA activation of full-length proGL-B is shown in Figure 3.13 (C,D). The molecular masses (M) of standard proteins are indicated. Incubation times (hrs) are shown above each gel.

Figure 3.15A SDS-PAGE analysis of progelatinase-B and proNGL-B activated by stromelysin



S = seconds

Figure 3.15B SDS-PAGE analysis of proNGL-B activated by APMA



(iii) Determination of k_{cat}/K_m values for gelatinase-B and NGL-B on QF24

The concentrations of the maximally activated species from 3.2:3 (ii) were determined by titration against the inhibitor TIMP-2 which unlike TIMP-1 does not interact with the proenzyme form (Murphy *et al.*, 1991).

Briefly, enzyme samples were preincubated for 2 hrs at 23°C in the presence of TIMP-2 in the concentration range of 2.5 nM - 40 nM prior to assay against QF24. Rates were determined over the first minute of the reaction over which time the linearity of the progress curve showed that no dissociation of enzyme - inhibitor complex had occurred. Active enzyme concentrations were calculated as described in Chapter 2.

Enzyme samples of known concentration were assayed against QF24 to determine values of k_{cat}/K_m. All assays were performed at 23°C in a total volume of 2.5 ml of assay buffer. The substrate concentration of 1.5 μM fulfilled the condition of $S << K_m$ required to allow direct determination of k_{cat}/K_m. Values for APMA and stromelysin activated progelatinase-B and proNGL-B were calculated as described in section 3.2:5 (iii). There were no significant differences between any of the activated species (Table 3.2), a result which is in agreement with similar studies performed using DnpPLGLWAR (data not shown).

Table 3.2

Characterisation of progelatinase-B activation

Enzyme	Activator	N-terminus	kcat/Km ($M^{-1}s^{-1}$) (QF24)
ProGL-B	APMA	M75	895,800
ProGL-B	STROMELYSIN	F88	895,460
ProNGL-B	APMA	M75	895,250
ProNGL-B	STROMELYSIN	F88	867,800

Activation conditions

The recombinant enzymes (2μM) were incubated at 37°C with either stromelysin (0.33μM) for 6 hours or APMA (2mM) for 22 hours. The N-terminii of the activated enzymes were determined as described in Chapter 2. Kinetic constants using QF24 were calculated as described in the text (3.2:3(iii)).

The progelatinase-B (ProGL-B) concentration was determined using an E_{280} value of 11,436 $M^{-1}cm^{-1}$ (by aminoacid analysis). The concentration of ProNGL-B was calculated using an E_{280} value of 71,246 $M^{-1}cm^{-1}$ by the method of Gill and von Hippel, (1989).

3.3: Discussion

This chapter has focused on three important biochemical properties of the gelatinases, namely, the events leading to enzyme activation, the specific activity of activated species and the role of the non-catalytic C-terminal domain. In conducting this work the author has endeavoured to define conditions which will be suitable for kinetic analysis of enzyme inhibitors and combined this with,wherever possible, studies that might indicate how these enzymes would function *in vivo*.

Gelatinase-A

Gelatinase-A is activated in the test-tube by APMA to produce an active form of 66 kDa with the aminoacid Y^{81} at the N-terminus. By showing that the process was not concentration dependent for stromelysin Okada *et al.* (1990) established that activation was initiated by an intramolecular cleavage. Two independent methods were used to examine this proposed mechanism of APMA activation with the following results;

(i) TIMP which can bind to stoichiometrically to gelatinase-A was not able to halt the conversion of pro- to active enzyme. This shows that the activation is not due to contaminating active enzyme and that the active-site is not available to bind to high molecular mass inhibitors. As this may be a result of steric hindrance a low molecular mass inhibitor (CT989) was also tested, with similar results.

(ii) Increasing the local concentration of gelatinase-A using heparin did not effect the rate of proenzyme disappearance but it did increase the rate at which the mature enzyme was produced.

In agreement with the studies of Okada (see Fig.1.4A), APMA promotes an intramolecular cleavage which is followed by a concentration dependent step leading to the formation of the mature enzyme.

APMA is unlikely to be the physiological activator of gelatinase-A although it is possible that it mimics endothelial cell stimulating angiogenic factor (ESAF) (Weiss *et al.*, 1983) or some other as yet undefined low molecular mass activator. There has been little progress in discovering a proteinase activator of gelatinase-A. *In vitro* studies indicate that serine proteinases such as plasmin and trypsin cannot bring about its activation and

this can be rationalised on the observation that gelatinase-A lacks a "bait" region which matches the specificity of these enzymes (Okada *et al.*, 1990; Strongin *et al.*, 1993). The matrix metalloproteinase, stromelysin has also been shown to be ineffective (Strongin *et al.*, 1993; Crabbe *et al.*, 1994b) although incubation with pre-activated gelatinase-A can result in activation (Crabbe *et al.*, 1994b). Several laboratories have reported that certain cells, particularly after induction with an agent such as concanavalin-A (Con-A), are capable of binding and activating gelatinase-A. Overall and Sodek (1990) reported that Con-A stimulated gingival fibroblasts produced increased levels of collagenase, matrilysin and gelatinase-A and furthermore the latter was fully active. Ward *et al.* (1991) have since shown that gelatinase-A activation by Con-A stimulated fibroblasts requires an intact C-terminal domain and is completely inhibited by TIMP-2 and partially by TIMP-1. Recently Strongin *et al.* (1993) identified an activation intermediate from HT1080 fibrosarcoma cells which was the product of a cleavage between residues N^{37} and L^{38}. This is not a serine proteinase recognition sequence but MMPs do cleave at such sites (section1.3:5). Accordingly, a range of MMPs were tested for their ability to activate progelatinase-A. Of these, fibroblast collagenase and matrilysin removed the propeptide from gelatinase-A and were, therefore, examined further.

Matrilysin cleaved progelatinase-A to the active species Y^{81} gelatinase-A but a transient intermediate could not be isolated in sufficient quantity to enable sequencing. Studies conducted by Crabbe *et al.* (1994a) using a catalytically dead mutant (E→A) demonstrated that matrilysin has the specificity to cleave progelatinase-A to the fully active form. Collagenase cleaved progelatinase-A at the N^{37} - L^{38} bond but was unable to complete the activation process. Removal of the remainder of the propeptide was completed by gelatinase-A itself. The rates of both collagenase and matrilysin induced gelatinase-A activation were augmented by the addition of heparin. Matrilysin does not, however, possess a recognisable heparin binding motif. Studies using gelatinase-A (E→A) (data not shown) proved that this was a result of enhanced gelatinase-A self-processing rather than matrilysin binding. Truncated gelatinase-A $_{(1\,-\,417)}$ was activated by collagenase at the same rate as its wild-type parent suggesting that activation was not preceded by a physical association of the two enzyme's C-terminal domains. Heparin had no

effect on the rate that collagenase activated the truncated enzyme, however, showing that this was a C-terminally mediated process. The action of heparin can be explained by its ability to bind to the C-terminal domain of both enzymes effectively concentrating them on its surface.

These results from these studies imply that collagenase or a related enzyme is responsible for the observations of cell-mediated progelatinase-A activation. The recent discovery of a membrane bound MMP which activates gelatinase-A suggests the latter (Sato *et al.*, 1994). Nonetheless, this does not preclude a role for collagenase in other circumstances. Mast cells, for example, are often found at sites of matrix remodelling where they release heparin and activators of procollagenase (Bromley and Wooley, 1984; Lees *et al.*, 1994).

Gelatinase-B

Progelatinase-B may be activated by a number of methods (Ogata *et al.*, 1992; Okada *et al.*, 1992) but the mechanisms involved in this activation are not fully understood. A comparative study was undertaken to analyse its activation by three different methods, APMA, trypsin and stromelysin. The contribution of the C-terminal domain to this process was studied using recombinant enzyme lacking this domain. Activated species were subsequently characterised by a combination of gel electrophoresis and N-terminal sequencing.

Both trypsin and APMA produced unproductive cleavages in the C-terminal domain of progelatinase-B during the activation process. Maximal activation by APMA was accompanied by the complete removal of this domain. Trypsin activation was complete after 7 hrs at which stage additional hydrolysis caused a time-dependent reduction in activity. The most efficient activator was stromelysin (6 hrs) which generated an 82 kDa species in a two stage process. In agreement with Ogata *et al.* (1992) stromelysin processing of wild-type progelatinase-B is largely confined to removal of the propeptide. Self-processing and activation of the wild-type and truncated enzymes occurred over a similar time-course in this study. Moreover, after active-site titration these were shown to have similar specific activities against peptide substrates (section 3.2:3 (iii)) and gelatin (data not shown). It can be concluded, therefore, that the C-terminal domain of progelatinase-B does not determine the

autoprocessing specificity of the catalytic domain nor the final substrate specificity. Interestingly, the continuing presence of the conserved cysteine in the APMA-activated enzymes did not modify the final specific activity (Table 3.2), thus, confirming the findings of Triebel *et al.* (1992). This group proposed a complex mechanism by which the thiol becomes involved in an internal disulphide rearrangement but there is as yet little evidence to substantiate this. Furthermore, binding of APMA to the cysteine may block subsequent reactions involving the thiol group.

Specific activity of gelatinases

A high enzyme specific activity (k_{cat}/K_m) is an important factor when attempting to determine inhibition constants (Ki's) in the range of 10^{-12} - 10^{-10} M because it permits reasonable reaction rates to be attained using a relatively low enzyme concentration (see Chapters 5 and 6). This permits the inhibitor concentration to be varied around its Ki without significant titration of the enzyme. k_{cat}/K_m values were determined for both gelatinase-A and gelatinase-B using three substrates in order to assess their utility for inhibitor studies.

The APMA-activated forms of both gelatinase-A and B were efficient against the peptide substrates, DnpPLGLWAR (Stack and Gray, 1989) and QF24 (Knight *et al.*, 1992). Both enzymes exhibited higher k_{cat}/K_m values against QF24, ranging from 10^{5} - 10^{6} $M^{-1}s^{-1}$, and there is some preliminary evidence to suggest that this is largely a consequence of an improved turnover rate (k_{cat}). This cannot be established definitively, however, since the lack of solubility of the QF24 substrate limits K_m measurements. Collagenase (Chapter 4) as well as stromelysin and matrilysin which are discussed later (Chapter 5) all exhibit comparatively low specific activities for these substrates (Murphy *et al.*, 1992b and data not shown).

Gelatinase-A also cleaves gelatin efficiently. The K_m for this substrate (1 μM) was considerably lower than that of either DnpPLGLWAR (70 μM) or QF24 (approximately 30 μM; Dr. F. Willenbrock, personal communication). Since gelatin is hydrolysed at a number of positions this value is, however, likely to reflect the contribution of many different cleavage sites. Consequently, gelatin is not a suitable substrate for kinetic analyses but its likeness to the

physiological substrate may provide a more rigorous test for inhibitors than the peptide substrates. The results of such analyses are described in Chapter 6.

Chapter 4

CHAPTER 4

CHARACTERISATION OF HUMAN RECOMBINANT INTERSTITIAL COLLAGENASE

4.1: Introduction

Interstitial collagenase is secreted as a 55 kDa non-glycosylated and a 57 kDA glycosylated proenzyme (Stricklin *et al.,* 1977). The mature enzyme has a molecular mass of between 42 kDa and 45 kDa depending on the mode of activation. These different activated species are known to exhibit varying degrees of activity against fibrillar collagen (Suzuki *et al.,* 1990). Moreover, collagenolytic activity is completely abolished upon removal of the C-terminal domain (Birkedal-Hansen *et al.,* 1988; Clark and Cawston, 1989; Murphy *et al.,* 1992b). In the following study, different forms of activated collagenase were generated by varying the activation protocol. These were then compared by measuring their specific activities against collagen and peptide substrates, with particular attention to the phenomenon of 'superactivation' which results from the inclusion of stromelysin in the reaction medium (Murphy *et al.,* 1987). This work was completed by assessing the contribution of the C-terminal domain to collagenase activity.

In common with most other members of this family, collagenase may be activated by AMPA or trypsin in the test-tube (Grant *et al.,* 1987; Nagase *et al.,* 1990; Suzuki *et al* ., 1990). Activation by trypsin starts with a cleavage at a K-R-R sequence in the propeptide. The identity of the final active form then appears to depend on the relative contributions of trypsin and collagenase to the progression of activation. APMA activation is characterised by an initial self-cleavage between valine (V^{67}) and methionine (M^{68}) five residues upstream from C^{73} (section 1.3:3). Prolonged incubation may lead to a further cleavage between phenylalanine (F^{81}) and valine (V^{82}) eight residues downstream of C^{73}. A number of groups have shown that the presence of stromelysin during the activation of collagenase by APMA or trypsin significantly augments its activity on its natural substrate, fibrillar collagen (Murphy *et al.,* 1987; Suzuki *et al.,* 1990). Activated collagenase which has retained C^{73} exhibits between 15 and 25 % of the collagenolytic activity of the stromelysin generated species. Prolonged exposure to APMA removes this cysteine to produce a species with 40 % of the

full activity. This shows that the formation of an enzyme with full collagenolytic activity requires more than just the removal of the cysteine.

In this study trypsin and stromelysin were used to produce different forms of collagenase that varied in the identity of their N-terminal amino acids. These were then investigated with respect to their activity on type 1 collagen and on peptide substrates. By analysing the properties of these enzymes it may be possible to establish if the stromelysin catalysed cleavage of the Q^{80}-F^{81} bond is crucial for the full expression of collagenase activity or, if there is some, as yet, unidentified mechanism by which stromelysin superactivates collagenase.

In Chapter 3, collagenase was shown to activate progelatinase-A. These studies indicated that the process may be reciprocated because trypsin-activated collagenase was observed to decrease in molecular mass during the reaction. This possibility was explored in a series of experiments leading to the characterisation of the activated collagenase by N-terminal sequencing.

The presence of an intact collagenase C-terminal domain has now been shown to be an absolute requirement for maintaining collagenolytic activity (Birkedal-Hansen *et al.,* 1988; Clark and Cawston, 1989; and Murphy *et al.,* 1992b). In order to characterise the contribution of the C-terminus to the biological activity of the enzyme a truncated version, $CL_{\Delta\ 243\ -\ 450}$ was studied. The role of the C-terminus and its potential as a target for the generation of specific inhibitors of collagenolysis was also tested by using a collagenolytic assay to identify C-terminal specific monoclonal antibodies which possessed neutralising activity.

4.2: Results

4.2:1 Collagenase activation

The following studies were designed to probe the so-called 'superactivation' of collagenase induced by stromelysin. This process has now been described by many groups (Murphy *et al.*, 1987; Suzuki *et al.*, 1990; Nagase *et al.*, 1990) but a detailed characterisation of the activated collagenase that includes its activity against peptide substrates has not been undertaken. The ability of gelatinase-A to superactivate collagenase is also examined.

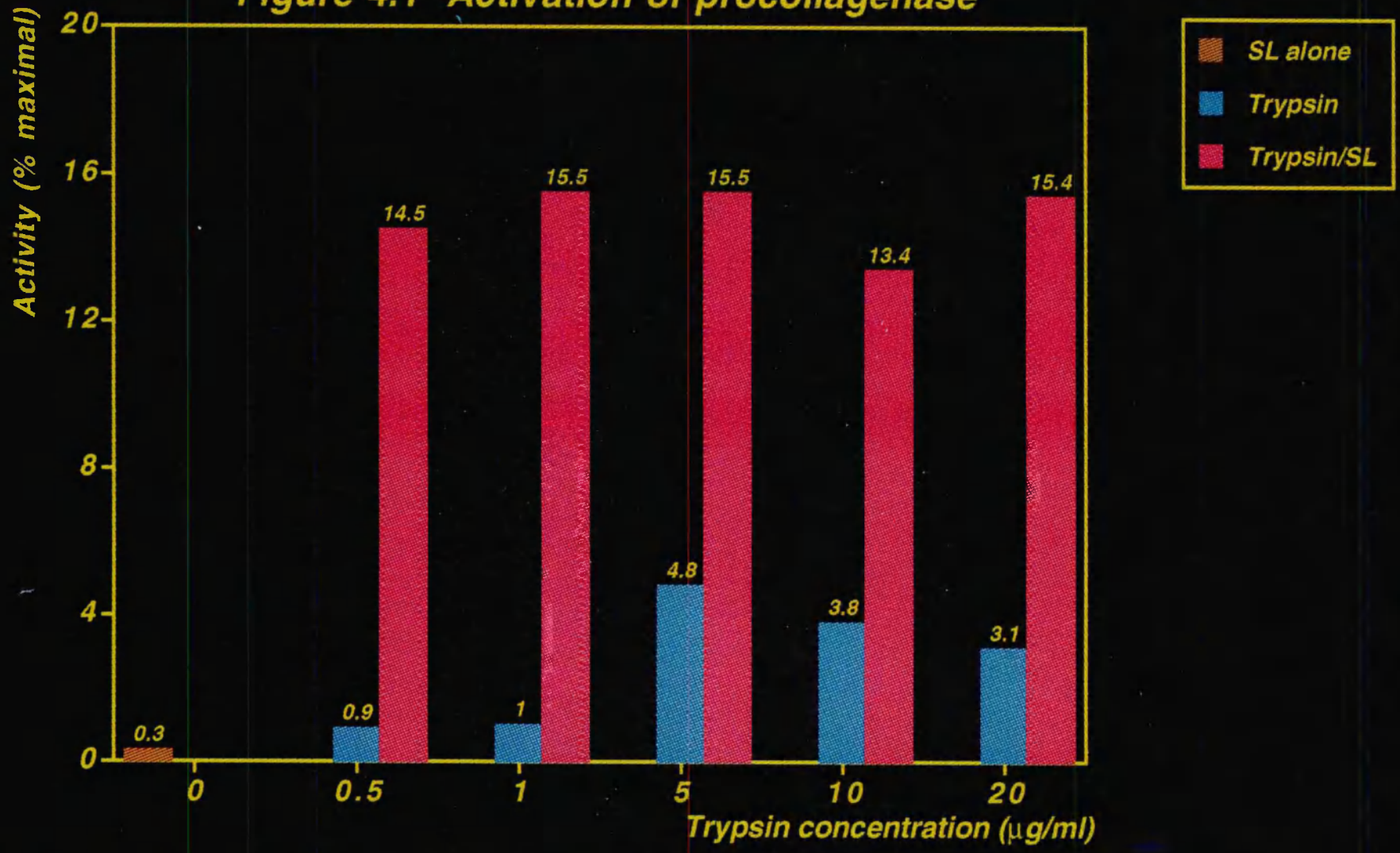
(i) The collagenolytic activity of human procollagenase activated by trypsin in the presence or absence of stromelysin

Procollagenase (2 μM) was incubated with a range of trypsin concentrations with or without stromelysin. The trypsin activity was terminated by adding excess soya bean trypsin inhibitor (SBTI) and then aliquots were tested for collagenolytic activity using the solution phase collagen assay described in Chapter 2. Fig. 4.1 shows the effect of stromelysin on trypsin activated samples. Stromelysin increased collagenase activity (expressed as percentage hydrolysis compared with a bacterial collagenase control) by between 4 and 20 fold over corresponding stromelysin negative samples. Gel electrophoresis of the products of this reaction showed that stromelysin was not acting synergistically with the collagenase to break down the collagen (data not shown). Increased collagenolytic activity is a result, therefore, of the action of stromelysin on collagenase.

(ii) SDS-PAGE anaiysis and N-terminal sequencing of coilagenase activated by trypsin, TPCK-treated trypsin and TPCK-treated trypsin in the presence of stromeiysin

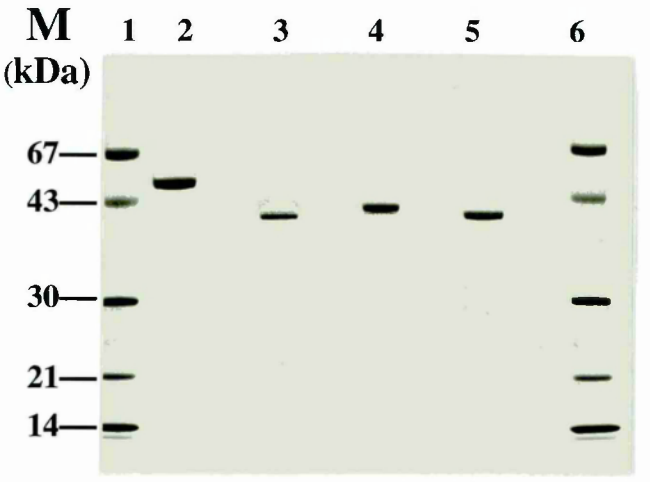
Trypsin is reported to cleave procollagenase at a number of sites (reviewed in Birkedal-Hansen *et al.*, 1993). The following experiment examines the enzyme species produced by trypsin in the absence and presence of an inhibitor, N-tosyl-L-phenylalanine chlormethyl ketone (TPCK), which blocks contaminating chymotryptic activity. These enzymes are compared to the active form of collagenase produced by a combination of trypsin and stromelysin.

Figure 4.1 Activation of procollagenase



Procollagenase (2µM) was incubated at 37°C for 30 minutes with increasing amounts of trypsin in the presence or absence of stromelysin (SL) (4µg/ml). Collagenolytic activity was determined using a glucose solubilised collagen assay as described in chapter 2. Values represent the mean of three readings.

Figure 4.2 SDS-PAGE analysis of procollagenase activation



Procollagenase (4μM) was incubated with trypsin or TPCK-treated trypsin in the presence and absence of stromelysin for 40 mins at 37°C as described in section 4.2:1 (ii)). Samples were then analysed by SDS-PAGE (10 - 20 %) under reducing conditions.

Lane	Sample
1	**Molecular mass markers**
2	**procollagenase**
3	**+ trypsin (10 μg/ml)**
4	**+ trypsin (TPCK) (10 μg/ml)**
5	**As above + prostromelysin (4 μg/ml)**
6	**Molecular mass markers**

Human procollagenase was incubated in the presence of trypsin (10 μg/ml)), or TPCK-treated trypsin with or without stromelysin (4 μg/ml) for 40 minutes at 37°C. Trypsin activity was terminated using a 10 fold excess of SBTI and the products were analysed by SDS-PAGE under reducing conditions essentially as described in Chapter 2 (Fig. 4.2).

Trypsin treated with TPCK produced a major species which migrated at a position corresponding to a molecular weight of 43 kDa (lane 4). The addition of stromelysin to this activation mixture resulted in a further reduction in molecular mass to 42 kDa (lane 5). A 43 kDa band was also produced by the action of untreated trypsin on procollagenase (lane 3).

N-terminal analysis of the activated collagenase was performed as described in Chapter 2 after electroblotting the appropriate band onto a polyvinylidine difluoride membrane. Table 4.1 shows the cleavage sites of the above activators together with the cleavages resulting from APMA activation of collagenase. Under the incubation conditions described above, TPCK-treated trypsin hydrolyses procollagenase at the bond between R^{72} and C^{73}. The residue,C^{73}, which contributes to the latency of the proenzyme by interacting with the catalytic zinc, is not removed by this method. Incubation of procollagenase with untreated trypsin resulted in an active enzyme form with V^{82} at the N-terminus. Inclusion of stromelysin in the TPCK-treated trypsin activation mixture resulted in the generation of an enzyme species with F^{81} at the N-terminus.

(iii) Characterisation of collagenase activation using the synthetic inhibitor CT435

Activation of procollagenase by any of the above methods may include self-cleavages like those produced by the inclusion of APMA in the reaction mixture. APMA is known to induce the hydrolysis of the F^{81} - V^{82} bond which, as shown above, can also be generated by incubating procollagenase with untreated trypsin. The ability of collagenase to self-activate was determined by including a synthetic collagenase inhibitor, CT435 (Fig. 5.1), in the reaction mixture. The products were analysed by SDS-PAGE (Fig. 4.3) and the results shown in Table 4.1. As would be predicted, CT435 blocked the activation of procollagenase by APMA showing that this is due to self-cleavage (lanes 7 and 8). CT435 did not affect the mobility of the active species generated by either

Table 4.1

Characterisation of collagenase activation

	Cleavage sites (↓)																									Inhibition of activation by CT435	Activity against collagen (%)	k_{cat}/K_m ($M^{-1}s^{-1}$) dnpPLGLWAR	k_{cat}/K_m ($M^{-1}s^{-1}$) QF24
								70										80											
1	....T		L	K	V		M	K	Q	P	R	↓	C	G	V	P	D	V	A	Q		F		V	L	T	NO	12	353	11,706
2	....T		L	K	V		M	K	Q	P	R		C	G	V	P	D	V	A	Q		F	↓	V	L	T	NO	17	902	36,440
3	....T		L	K	V		M	K	Q	P	R		C	G	V	P	D	V	A	Q	↓	F		V	L	T	YES	100	827	16,102
4	....T	↓	L	K	V	↓	M	K	Q	P	R		C	G	V	P	D	V	A	Q		F	↓	V	L	T	YES	13	ND	ND

Activation conditions

1 **TPCK - treated trypsin (1:40 w:w trypsin to collagenase),40 mins at 37°C**

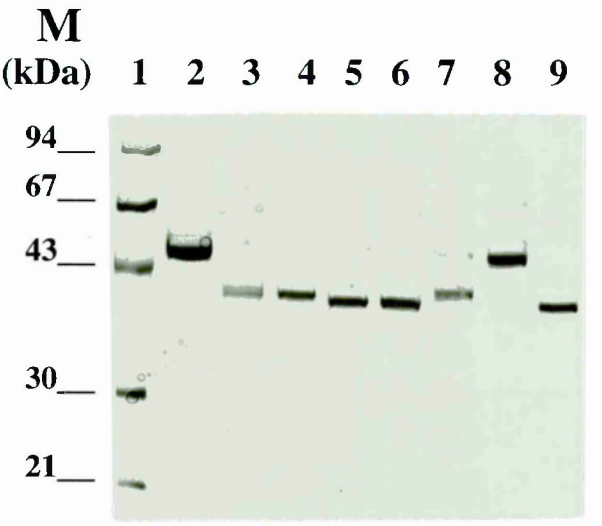
2 **Untreated trypsin as above**

3 **TPCK - treated trypsin (1:40 w:w) + stromelysin (1:50 w:w),40 mins at 37°C**

4 **APMA (1mM),60 mins at 37°C**

Experimental details are described in the text (section 4.2:1 (ii - iv)). The N-terminal sequences of the fully activated forms of the enzyme are high-lighted.

Figure 4.3 Inhibition of the activation of procollagenase using CT435



Procollagenase (4 μM) was incubated for 40 mins at 37°C in the presence of activating agent ± the synthetic inhibitor CT435. Details of each reaction are given below. Samples were then analysed by SDS-PAGE (10 - 20%) under reducing conditions.

Lane	Sample
1	**Molecular mass markers**
2	**Procollagenase (ProCL)**
3	**ProCL + TPCK-treated trypsin (10 μg/ml)**
4	**As (3) + CT435 (15 μM)**
5	**ProCL + trypsin (10 μg/ml)**
6	**As (5) + CT435 (15 μM)**
7	**ProCL + APMA (0.5 mM)**
8	**As (7) + CT435 (15 μM)**
9	**ProCL + trypsin (10 μg/ml) + stromelysin (4 μg/ml) control**

TPCK-treated (lanes 3 and 4) or untreated trypsin (lanes 5 and 6) suggesting that autocatalysis is not involved in these processes. Moreover, the generation of the N-terminal residue V^{82} by untreated trypsin must be due to a component of the trypsin mixture itself such as chymotrypsin rather than the action of collagenase which would be inhibited by CT435. The F^{81} - V^{82} bond does not appear, therefore, to be a collagenase self-cleavage site in the absence of a structural perturbant such as APMA.

(iv) Characterisation of collagenase activation using specific activity measurements

The specific activity of trypsin and trypsin / stromelysin activated collagenase was measured against collagen and two synthetic substrates, DnpPLGLWAR and QF24. Concentrations of active enzyme were determined by active site titration against TIMP-1 as described in section 2.2:5.

Collagenolytic activity was measured using semi-fibrillar collagen in a diffuse fibril assay as described in section 2.2:6. Table 4.1 shows the activity of each form (together with published data for the APMA activated enzyme (Nagase *et al.,* 1990)) expressed as a percentage of that produced by trypsin/stromelysin (F^{81}-collagenase). Values for k_{cat}/K_m were generated for the two peptide substrates as described in Chapter 2 under conditions of $S << K_m$ required to allow the direct determination of this property (Table 4.1).

Collagenase with the C^{73} N-terminus produced by the action of TPCK-treated trypsin possessed only a fraction of the collagenolytic activity associated with stromelysin "superactivation". This level of activity was comparable to that obtained by Nagase *et al.* (1990) for APMA activated collagenase (13%). (C^{73})-Collagenase also expressed comparatively low activity against DnpPLGLWAR and QF24 (Table 3.2).

(V^{82})-Collagenase produced by the action of untreated trypsin, possessed only 17% of the collagenolytic activity expressed by (F^{81})-collagenase. Specific activity against the peptide substrates as indicated by the k_{cat}/K_m value was equivalent or improved for the (V^{82})-collagenase compared to the (F^{81}) form of the enzyme (Table 4.1).

The data suggest that removal of C^{73} is a requirement for the expression of full activity against both the physiological substrate and small synthetic peptides. Removal of F^{81} from the N-terminus of the active enzyme significantly

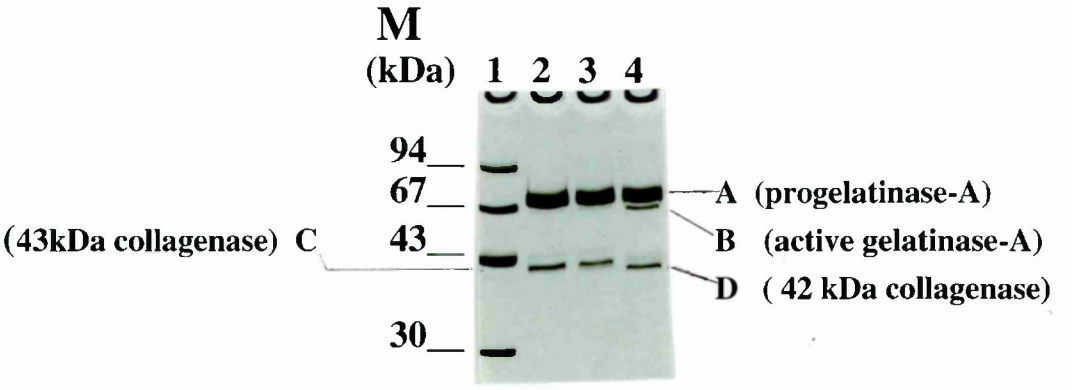
reduces its ability to hydrolyse collagen whilst activity against peptide substrates appears to be maintained or improved. This suggests that F^{81} may be involved in collagen recognition. Its presence does not appear to be necessary for the hydrolysis of small peptides.

(vi) Activation of procollagenase by gelatinase-A

Stromelysin-1, stromelysin-2 and matrilysin have been reported to superactivate collagenase (Murphy *et al.,* 1987; He *et al.,* 1989; Nicholson *et al.,* 1989; Quantin *et al.,* 1989). There is no evidence to date to suggest that gelatinase-A possesses this capability even though it is often found at sites of tumour invasion together with collagenase. The following experiment examines the effect of gelatinase-A on collagenase activation. A related series of experiments describing the action of collagenase on gelatinase-A activation were presented in section 3.2:1.

Progelatinase-A was added to trypsin-activated collagenase in gelatinase activation buffer. The reaction mixture was incubated at 37°C and at times ranging from 0 - 6 hours an aliquot was removed and examined by SDS-PAGE under reducing conditions (Fig. 4.4). At time 0 hrs the trypsin activated collagenase can be seen as a band migrating with an apparent molecular mass of 43 kDa. After 2 hours a single band of 42 kDa can be observed. The production of this band is accompanied by the concomitant increase of a 66 kDa species which, as shown earlier, corresponds to activated gelatinase-A. Bands representing the 43 kDa form (0 hours) and the 42 kDa form (2 hours) of collagenase were N-terminally sequenced following electroblotting onto polyvinylidine difluoride membranes as described in section 2.3:2. Collagenase activated by trypsin alone was comprised largely of a form with C^{73} at its N-terminus. Incubation of the trypsin-activated collagenase with progelatinase-A resulted in the production of a band of lower molecular mass with an N-terminus corresponding to F^{81}. This is the same N-terminal residue that is produced by the action of stromelysin, which was shown previously to correlate with 'superactivation' of collagenase.

Figure 4.4 Activation of procollagenase by gelatinase-A

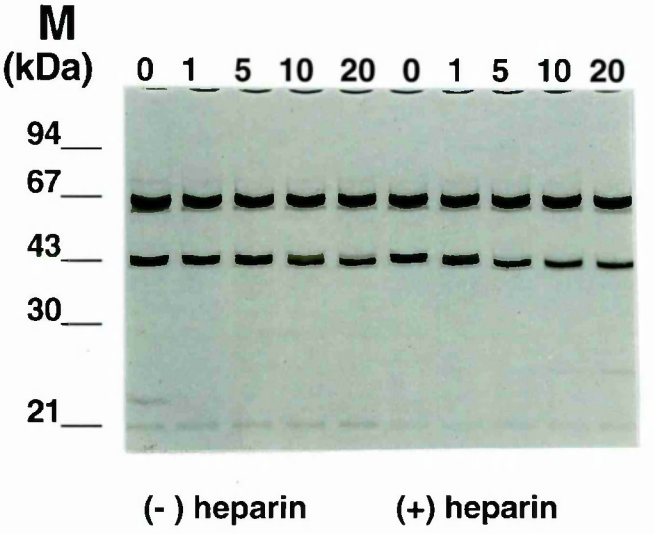


Procollagenase (2μM) was activated with TPCK-treated trypsin (0.4μM) for 45 minutes at 37°C. Trypsin activity was terminated by the addition of a 10 fold excess of SBTI. This solution was then incubated at 37°C with progelatinase-A in a total assay volume giving enzyme concentrations of 1μM and 4μM for collagenase and gelatinase, respectively. Aliquots were removed from each sample for analysis on a 10 - 20% polyacrylamide gel under reducing conditions.

Lane	Sample
1	Molecular mass markers
2	0 hours
3	1 hour
4	2 hours

Band C (0 hrs) and band D (2 hrs) were electroblotted onto a polyvinylidine fluoride membrane and then N-terminal sequenced as described in Chapter 2.

Figure 4.5 Time-course of gelatinase-A activation of procollagenase: The effect of heparin



Procollagenase (0.75 μM) was incubated with an equimolar solution of gelatinase-A at 37°C in the presence or absence of heparin (100 μg/ml). At 0, 1, 5, 10 and 20 minutes, 20 μl was removed and then analysed by SDS-PAGE (10 - 20%) under reducing conditions (incubation times are shown above each lane). Molecular masses (M) of standard proteins are indicated.

(vii) The rate of superactivation of collagenase by gelatinase-A: the effect of heparin

Previous studies have shown that gelatinase-A is capable of superactivating collagenase. The efficiency of this process may be tested by following the conversion using SDS-PAGE. Moreover, heparin may increase the efficiency of the reaction in a comparable process to that described earlier for the activation of progelatinase-A by collagenase (Chapter 3).

Trypsin-activated collagenase was incubated at 37°C with an equimolar concentration of gelatinase-A in the presence or absence of heparin. At various times an aliquot was removed and then analysed by SDS-PAGE under reducing conditions (Fig. 4.5). Superactivation is associated with a reduction in molecular mass of about 1 kDa. Figure 4.5 shows that in the absence of heparin the conversion is nearly complete after 20 minutes (lane 6). In the presence of heparin the reaction is completed by between 1 (lane 8) and 5 minutes (lane 9).

Stromelysin, at a stromelysin:collagenase ratio of 1:100 (M:M) completes the superactivation process within 10 minutes (unpublished data). This study indicates, that in the absence of heparin, superactivation by gelatinase-A is of the order of 200 fold less efficient. Addition of heparin,however, increases this efficiency by 10 fold whereas it does not affect the stromelysin catalysed reaction (data not shown).

3.2:2 The role of the C-terminal domain of collagenase

The C-terminal domain of collagenase does not possess any inherent catalytic activity but may, by binding to the collagen molecule, expose a site for cleavage by the catalytic domain.

The importance of this domain was established by two methods. Firstly, a monoclonal antibody (MAC065) was shown by western blotting to bind to a region within the collagenase C-terminal domain. Its effect on collagenolysis and collagenase activity against a peptide substrate were then studied. Secondly, a genetically engineered collagenase ($\Delta_{243\text{-}450}$ collagenase) which lacked the entire C-terminal domain was compared to full-length enzyme with respect to TIMP-1 binding, collagenolysis and specific activity against a peptide substrate. Results arising from studies on the truncated collagenase are summarised in Table 4.2.

(i) Screening monoclonal antibodies using the collagen diffuse fibril assay

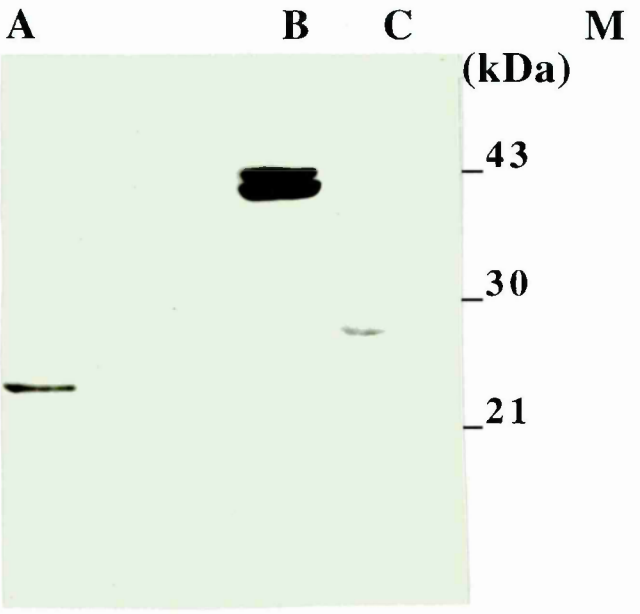
A selection of murine monoclonal antibodies (MAbs), that had been raised against both recombinant human and dog collagenase, were assayed for their ability to inhibit collagenolysis using the diffuse fibril assay (Chapter 2).

Antibodies were first screened at concentrations of 0.1μM and 1μM and the extent of inhibition was determined as a percentage of the activity of a collagenase alone control. Three MAbs MAC064, MAC065 and MAC066, showed 40%,100% and 50% inhibition respectively, at a concentration of 0.1μM. Mac065 was re-tested at a range of concentrations. An IC_{50} value of 3nM was determined from a plot of percentage inhibition (y - axis) against Log_{10} [MACO65] (x - axis) (section 2.2:6).

(ii) Western blotting of MAC065 to determine its domain specificity

The C-terminal domain of collagenase (Δ 1-261) was generated by incubating collagenase with stromelysin and trypsin as described above, in the presence of 0.8 mg/ml 1,10-phenanthroline. This method produces a single band of 30 kDa which has been identified by N-terminal sequencing as the C-terminal domain beginning at residue I^{262}. The sample was concentrated two fold using an ultrafree concentration unit with a molecular mass cut-off of 10 kDa and then analysed by gel electrophoresis under both reducing and non-reducing conditions. Identical samples were run on both halves of the gel. One half of the gel was stained with Coomassie-Blue whilst the other half was western blotted using an HRP-linked second antibody as described in Chapter 2. Enhanced chemiluminescence (ECL) was used to visualise HRP containing bands (Fig. 4.6.), which were compared with equivalent Coomassie-Blue stained samples. Intense staining was associated with bands corresponding to reduced full-length active collagenase and both the reduced and non-reduced C-terminal domain of collagenase. Other studies have shown that MAC065 does not recognise the catalytic domain of collagenase (data not shown). This indicates that the epitope for MAC065 resides in the C-terminal domain of collagenase. The observation that the antibody recognised reduced C-terminal domain would suggest that this epitope is a linear sequence of amino acids.

Figure 4.6 Characterisation of the specificity of MAC065 using western blotting



Lane	Sample
A	Non-reduced C-terminal domain
B	Active collagenase
C	Reduced C-terminal domain

Collagenase C-terminal domain ($\Delta_{1\text{-}211}$)CL was generated by incubating procollagenase (2μM) with TPCK-treated trypsin (10μg/ml) and stromelysin (4μg/ml) for 45 minutes at 37°C in the presence of the zinc binding agent 1, 10 - phenanthroline (0.8mg/ml). The identity of the C-terminal domain was determined by N-terminal sequencing. Samples were electrophoresed on a 10-20% polyacrylamide gel and then transferred to an immobilon membrane by electroblotting (30 volts) for 16 hours. The samples were western blotted using MAC065 as the first layer as described in the text (4.2:2 (ii)). Proteins that were recognised by MAC065 were visualised by ECL detection (Chapter 2). The molecular masses (M) of standard proteins are indicated.

(iii) MAC065 inhibition of collagenase activity on DnpPLGLWAR

The presence of an intact C-terminal domain on collagenase is not required for cleavage of DnpPLGLWAR (section 3.2:4{v}). Consequently, an antibody directed against this region would not be expected to neutralise the enzymic activity of collagenase on such a substrate. The assay may, therefore, be used to discriminate between those agents which interfere with the catalytic activity of the enzyme and those that bind to enzyme but do not directly effect catalysis.

Collagenase (200 μg/ml) was activated by incubating it with trypsin (4 μg/ml) and stromelysin (4 μg/ml) at 37°C for 40 minutes. The collagenase mixture was diluted to a final concentration of 10 μg/ml for assay. MAC065 was tested at a concentration of 1μM for its ability to inhibit the activity of collagenase against DnpPLGLWAR. The reactants were mixed in peptide assay buffer to a final volume of 135 μl at 23°C. The reaction was initiated by the addition of 15μl of DnpPLGLWAR (1mM) resulting in a final substrate concentration of 100μM. At times between 0 mins and 30 mins an aliquot (20 μl) was removed for fluorescence measurement. Duplicate samples containing MAC065 gave rates of 14.5 and 11.1 FU/min. These rates are not significantly different from the enzyme alone controls (12.9 and 13.4 FU/min). These results show that MAC065 does not neutralise the catalytic properties of collagenase and that the antibody probably inhibits collagenolysis by masking the enzyme's collagen binding site.

(iv) Characterisation of $\Delta_{243\text{-}450}$ collagenase : Inhibition by TIMP-1

An apparent Ki (Ki_{app}) for the inhibition of full-length and $\Delta_{243\text{-}450}$ collagenase by TIMP-1 was determined by assaying against DnpPLGLWAR. The assay was performed using a substrate concentration of 20μM, an enzyme concentration of 2 - 5 nM and TIMP-1 concentrations in the range of 0.4 - 22 nM. Overnight assays were performed at 37°C with timepoints of 0 and 16 hrs. Results were analysed using Enzfitter (Leatherbarrow, 1987) by a nonlinear regression analysis of the equation for tight-binding inhibition described by Morrison and Walsh (1988) (Chapter 2).

The results for full-length collagenase were of limited accuracy, since enzyme activity was titrated by TIMP-1 even at the lowest enzyme concentration of 2nM. The Ki_{app} is, therefore, less than 0.2nM and could not be analysed

Table 4.2

Characterisation of collagenase and Δ243-450 collagenase

Enzyme	Collagen (μg/ml)	DnpPLGLWAR k_{cat}/K_m ($M^{-1}s^{-1}$)	TIMP-1 Ki (nM)
Collagenase	97.9	827	<0.2
Δ243-450 Collagenase	0	653	1.2

The recombinant human enzymes were activated by incubation with trypsin (10 μg/ml) and stromelysin (4 μg/ml) for 45 minutes at 37°C. Under these conditions the contribution of stromelysin to the rate of substrate hydrolysis or to the association of collagenase with TIMP-1 is not significant. Collagenase concentrations were determined by titration against TIMP-1 as described in Chapter 2. Assays were conducted as described in the text (4.2:2 {iv - vi}).

further owing to limitations in the sensitivity of the assay. A Ki_{app} value of 1.2 nM was obtained for the truncated enzyme and this figure was reproducible over the course of several experiments (Table 4.2). The results indicate that the C-terminal domain of collagenase is involved in the interaction of the enzyme with TIMP-1.

(v) k_{cat}/K_m determinations

Collagenase and $\Delta_{243-450}$ collagenase were assayed against DnpPLGLWAR. The K_m was determined to be greater than 300μM which means that the substrate concentration of 20 μM fulfilled the condition of $S << K_m$ required to allow the direct determination of k_{cat}/K_m. Assays were performed at 37°C in peptide assay buffer using 30 - 60 nM enzyme. At time intervals up to 1hr, 20 μi aliquots were withdrawn and added to 500 μl of stopping buffer. Reaction rates were determined as described in Chapter 2. The k_{cat}/K_m values for the two enzymes were not significantly different (Table 4.2) suggesting that the C-terminal domain of collagenase does not influence the catalytic activity of the enzyme..

(vi) Collagenolytic activity

Collagenolytic activity was assayed using the $[^{14}C]$ - collagen diffuse-fibril assay as described in section 2.2:6. The truncated enzyme showed no collagenolytic activity at the test concentration whilst full-length collagenase cleaved collagen at a rate of 97.9 μg/min/ml. The specific activity of full-length collagenase has been observed to be variable and it rises as the solubility of the collagen used in the assay is increased. The results (Table 4.2) show that the C-terminal domain of collagenase is an essential requirement for the hydrolysis of type 1 collagen.

4.3: Discussion

Human interstitial collagenase can be activated by both APMA and trypsin. Murphy *et al.* (1987) showed that addition of stromelysin to activated collagenase produced a form of collagenase with a higher specific activity against collagen. A comparative study of these different forms of collagenase has not, however, been performed using peptide substrates. Moreover, there is still some debate over the explanation for the improved collagenolytic activity of the 'superactivated' collagenase.

In this study, collagenases which differed in their amino-terminal sequences were tested against collagen and two peptide substrates. The ability of gelatinase-A to superactivate collagenase was also examined and the contribution of the C-terminal domain to collagenase activity and inhibition was studied using a genetically engineered enzyme.

The superactivation of collagenase by stromelysin

Incubation of procollagenase with TPCK-treated trypsin produces an active 43 kDa form of collagenase with the amino-terminus C^{73}. Addition of stromelysin removes a further eight residues generating a form which starts at residue F^{81}. This species is several fold more active on collagen and twice as active against peptide substrates. These results are consistent with those of Knauper *et al.* (1993) who showed that the specific activity of trypsin-treated neutrophil collagenase against a peptide substrate was improved after the addition of stromelysin. Removal of the peptide containing C^{73}, therefore, enhances the catalytic activity of collagenase. (V^{82})-Collagenase can be formed by the action of trypsin which has not been treated to remove chymotryptic activity. That this species was not a product of self-cleavage was demonstrated by its lack of sensitivity to CT435, a collagenase inhibitor. Surprisingly, this form of collagenase is also less active than superactivated collagenase against collagen but its activity on peptide substrates is at least comparable (Table 4.1). There has been some debate on the nature of superactivation since stromelysin generated collagenase differs by only one residue from that produced by APMA. One explanation is that it is merely an artifact of the assay system. The collagen

assay is dependent upon differential precipitation to separate the hydrolysed fragments from intact collagen. Additional cleavages within these fragments may produce an apparent increase in the level of soluble material which can be wrongly attributed to the action of collagenase. The prospect that synergism between the two enzymes was responsible for increased collagen degradation was investigated by studying the collagen cleavage products using SDS-PAGE (data not shown). These results indicated that 'superactivation' leads to a genuine increase in the collagenolytic potential of collagenase.

The crystal structure of the superactivated form of the catalytic domain of neutrophil collagenase has recently been solved by Reinemer *et al.* (1994). They concluded that by making a salt link with a C-terminal aspartic acid (D^{232}), the amino-terminal phenylalanine promotes a more ordered structure of the N-terminal fragment. It is argued that the reduction in mobility of this fragment might then prevent it from entering the substrate binding cleft of another molecule thus blocking its activity. This study, however, provided no evidence that the catalytic activity of fibroblast collagenase is modified. An alternative proposal is that the salt bridge involving D^{232} helps provide the correct juxtaposition of the catalytic domain and the C-terminal collagen binding domain. In this way only collagenolytic activity would be affected.

The superactivation of collagenase by gelatinase-A

Superactivation may explain the accelerated degradation of collagens observed under certain physiological and pathological conditions (Unemori *et al.*, 1991) but stromelysin, having a low pH optimum (Harrison *et al.*, 1992), would be expected to retain only a fraction of its activity outside the acidic environment of the joint matrix. It is evident from reports of matrilysin's capacity to carry out the Q^{80} - F^{81} cleavage (Quantin *et al.*, 1989) that alternative means of superactivation exist. During studies on the activation of progelatinase-A by collagenase (Chapter 3) a reduction in the molecular mass of the collagenase was noted. The newly formed collagenase was subsequently identified as the F^{81} form by N-terminal sequencing. This establishes that like stromelysin and matrilysin, gelatinase-A is also capable of superactivating collagenase. The process was, however, about 200 fold less efficient than that due to stromelysin although the difference was less substantial (20 fold) in the presence of heparin.

The role of the C-terminal domain

The activity of a genetically engineered mutant which lacks the C-terminal domain was tested against collagen and a peptide substrate. In agreement with Birkedal-Hansen *et al.* (1988) and Clark and Cawston, (1989) the collagenolytic activity of the truncated collagenase was totally abolished. In contrast, activity against a fluorescent peptide substrate was not significantly modified. This shows that collagen is only recognised as a substrate by collagenase after binding of the enzyme C-terminal domain modifies its structure. Indeed, collagen modelling experiments indicate that only one chain of the triple-helical collagen molecule can be accommodated in the active-site cleft at any one time (Bode *et al.*, 1994). This suggests that the role of the C-terminal domain is to promote the unravelling of the triple helix at the cleavage site, thus exposing it for cleavage.

The C-terminal domain also increases the strength of binding of TIMP-1 to collagenase. In its absence the Ki for this interaction is of the order of 10^{-9} M which is similar to that for the naturally truncated MMP, matrilysin (unpublished data). Assays lacked the sensitivity to determine an accurate Ki for the full-length form of collagenase but it is likely to be lower than 10^{-10} M.

The proposal that the C-terminal binding interaction might be a target for inhibitors was tested using specific monoclonal antibodies (MAbs). MAC065, which did not neutralise the activity of collagenase against a peptide substrate, proved to be highly efficient in blocking collagenolysis. Clearly, inhibition of collagen binding presents an opportunity to discover highly selective inhibitors of collagenase.

In summary, both stromelysin and gelatinase-A can process collagenase to a form which is fully active against collagen. The increased collagenolytic activity correlates with the appearance of F^{81} at the N-terminus. Interestingly, the effect is collagen specific since a form of collagenase with the phenylalanine removed, retains activity against peptide substrates. In Chapter 3 gelatinase-A was shown to be processed by collagenase. The reciprocated activation of these two enzymes which is enhanced in the presence of heparin may be physiologically relevant to mast cell action.

Collagenase lacking a C-terminal domain does not cleave collagen, but this domain is not required for peptidase activity. Inhibition of the binding of this domain to collagen would selectively block collagenolysis without affecting other

activities of collagenase. Moreover, the site responsible for collagen binding is presumably absent in other MMPs making it a potentially selective target for intervening in pathological collagen breakdown.

Chapter 5

CHAPTER 5

THE DESIGN OF POTENT SYNTHETIC MMP INHIBITORS

5.1: Introduction

In Chapters 3 and 4 the biochemical characterisation of gelatinase-A, gelatinase-B and collagenase was described. These enzymes together with stromelysin and matrilysin were then used to test the efficiency of inhibitors synthesised by the Celltech Chemistry Department. The focus of the chemistry effort towards the design of first generation compounds has been targeted at synthesising competitive, reversible inhibitors, which prevent substrate hydrolysis by binding to the active site of gelatinase-A. In this chapter, data will be presented describing our use of structure activity relationships (SARs) for rational drug design.

The first orally active drug against a zinc metalloproteinase was the ACE inhibitor, captopril (Fig. 1.8). This was designed on the basis of the structure of the active site of the zinc exopeptidase, carboxypeptidase A (Ondetti *et al.* 1977; Cushman *et al.* 1977). Its strength of binding (Ki = 1.7 nM) derives largely from a sulphydryl group (-SH) which mimics the carbonyl oxygen of the scissile bond and binds to the zinc atom at the active site. Additional binding and specificity is provided by a pseudo-peptide structure which has features of bradykinin, an ACE substrate. Subsequently, a variety of zinc binding ligands have been incorporated into molecules of this basic design. This work has resulted in the synthesis of carboxylic acid (-COOH) (Patchett *et al.*,1980), phosphonic acid (eg. $-PO(OH)_2$) (Holmquist and Vallee, 1979; Galardy, 1980) and hydroxamic acid (-CONHOH) (Subissi *et al.*, 1992) inhibitors with nanomolar or sub-nanomolar Ki's.

The design of MMP inhibitors has not been based on crystal structures since until recently these have not been available. The first rational design of collagenase inhibitors was reported by Gray *et al.* (1981) who used a similar approach to that used successfully for the ACE inhibitors. This right hand side (RHS) inhibitor had an IC_{50} of 10 μM against tadpole collagenase. By 1986 a range of zinc-binding ligands had been designed (See Chapter 1) the best of which was a Searle hydroxamic acid (Dickens *et al.*, 1986) (Fig. 1.9) with an IC_{50}

of 20 nM against human interstitial collagenase. Two other important requirements for efficient inhibition had also been established,

1. Collagenase is stereoselective with a preference for R,S,S stereochemistry at the P_1', P_2' and P_3' binding sites, respectively.
2. Inhibitors based on the left hand side (LHS) of the cleavage site are significantly less active than their RHS counterparts.

The compound Z-Pro-Leu-Gly-NHOH, for example, had an IC_{50} of 40 μM against collagenase despite the presence of a hydroxamate ligand.

Celltech compounds are classified as RHS inhibitors and are structurally related to the Searle inhibitor described above. The relationship of the structure to efficiency and selectivity was examined with the aid of structurally related compounds. In this way the consequence of a single modification could be analysed. Structure-activity relationships (SARs) were constructed for gelatinase-A, stromelysin and collagenase and selected compounds were also tested against gelatinase-B and matrilysin. Selectivity data were also obtained for representatives of other zinc metalloproteinase families, ACE and aminopeptidase-N (AP-N). In early studies results were expressed as IC_{50}'s (section 1.4:5). The introduction of peptide based assays allows Ki's to be calculated in later studies. SAR results will be presented in the form of Ki's where the experimental design permits. The following studies were designed to identify the optimum zinc binding ligand together with the side-chains on the pseudo-peptide backbone (P_1' - P_3'/P_4') which are important for gelatinase binding and discriminate against other enzymes. Finally this information is summarised in the form of an inhibitor model based on crystal structure data.

5.2 : Results

5.2:1 The Design of Selective Gelatinase inhibitors from experimental SARs

A series of compounds were chemically synthesised of the form **Z - P_1' - P_2' - P_3'** in which **Z** represents a zinc binding ligand and **P_1'**, **P_2'** and **P_3'** align with the corresponding **S_1'**, **S_2'** and **S_3'** substrate binding sites of the enzyme. SARs were then constructed to allow the contribution of each inhibitor side-chain to be examined. Inhibitor efficiency, expressed as Ki's, was determined using DnpPLGLWAR. Particularly active gelatinase-A inhibitors (Ki < 0.05 nM) were also analysed using the more sensitive substrate, QF24.

Mechanistic studies have revealed that many of the compounds exhibit time-dependent inhibition at concentrations used for Ki determination. An example of this will be presented later (section 6.2:2 {c}). In order to minimise the effects of time-dependent inhibition, enzymes were routinely pre-incubated for up to 4 hours in the presence of inhibitor, prior to the addition of substrate. Apparent Ki's (Ki_{app}) were determined from steady-state rates as described in Chapter 2. Ki's were determined using the relationship, $\mathbf{Ki_{app} = Ki(1 + {}^{S}/_{Km})}$ (competitive inhibition). Since, collagenase and stromelysin assays were performed under conditions of $S << K_m$, Ki_{app} is equivalent to the true Ki. A K_m value of 70 μM was determined for the hydrolysis of DnpPLGLWAR by gelatinase-A (Chapter 3) and substitution of this value in the above equation permits Ki_{app} values to be converted to Ki's. Hydrolysis of QF24 by gelatinase-A was monitored continuously using a substrate concentration significantly lower than the K_m. Data was then analysed as described in Chapter 2.

(i) Analysis of the zinc binding group (Table 5.1)

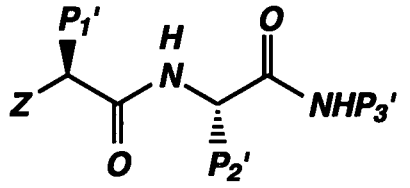
A variety of compounds with modifications of the zinc binding ligand were synthesised by the Celltech Chemistry Department. Ki values were established as described above and Table 5.1 shows the results for a representative selection of these inhibitors.

Compounds with a hydroxamic acid (-CONHOH) ligand (CT572, CT921, CT471 and CT989) proved to be far more effective against all test enzymes than inhibitors with alternative ligands. The rank order potency for gelatinase-A and stromelysin is in the order of -CONHOH >> -COOH ≥ $-PO(OH)_2$ >> -SH. Only

Table 5.1

Modifications of the Zinc-Binding Ligand

General Structure



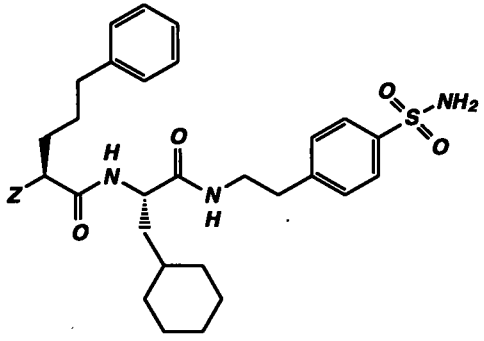
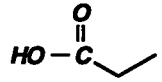
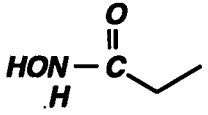
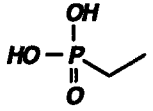
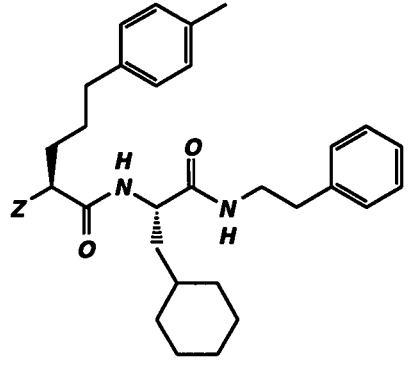
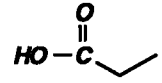
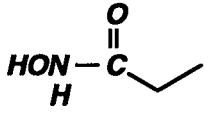
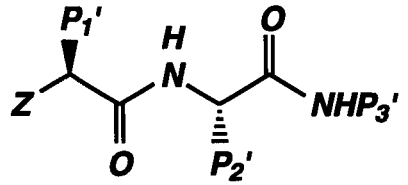
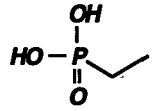
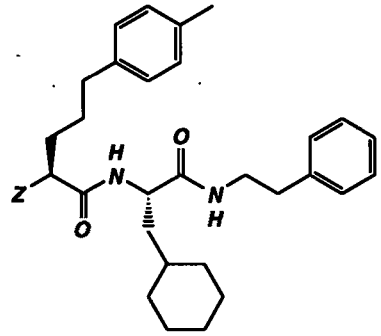
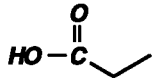
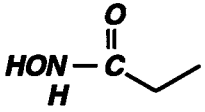
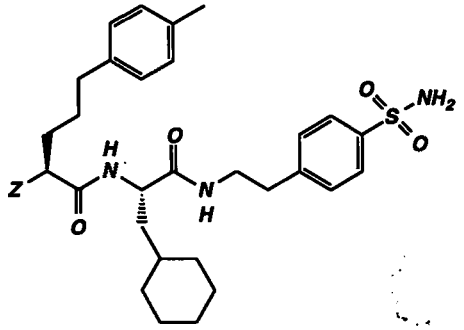
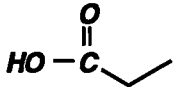
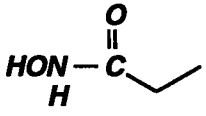
CT Number	Z		ACTIVITY / Ki [nM] GL-A	SL	CL
730			15,300	23,000	37,000
701			6.4	9.52	40,000
572			0.02	5.9	150
1167			2.5	277	17,000
876			0.99	472	22,100
921			0.08	8.2	921

Table 5.1 Cont'd

Modifications of the Zinc-Binding Ligand

General Structure



CT Number	Z		ACTIVITY / Ki [nM] GL-A	SL	CL
940			18.9	701	25,000
548			21	842	44,700
471			0.06	8.3	203
1466	No Z Group		2,670	>100,000	>100,000
976			0.9	384	47,800
989			<0.01	2.98	329

the presence of hydroxamic acid produced sub-micromolar inhibition of collagenase. In some cases substitution of -CONHOH with -COOH did appear to improve selectivity for gelatinase-A over collagenase (compare CT921 {11,500} and CT876 {22,322}) but this relationship was not consistent for all series studied. Similarly, phosphonic acid ligands had no consistent effect on the SARs for each enzyme. In all cases activity against gelatinase-A was substantially reduced in comparison to the corresponding hydroxamic acid inhibitor. Replacement of the hydroxamic acid with a thiol group (CT572 , CT730, respectively) caused a dramatic loss in potency against gelatinase-A in particular. Selectivity was also abolished suggesting that the thiol was not interacting with the zinc either as a consequence of poor positioning or of oxidation. This is substantiated by the observation of a reduced Ki after complete removal of the zinc binding ligand (CT1466). Removal of the zinc-binding ligand reduced activity by the order of 10^5 to 10^6 over the corresponding hydroxamate (CT989). Nonetheless, the Ki value of 2.67 μM for CT1466 is much lower than the K_m of any published peptide substrate indicating a reasonable fit of the peptide side-chains into the binding pockets of the enzyme.

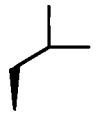
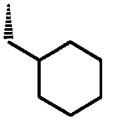
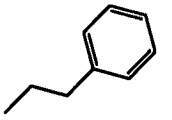
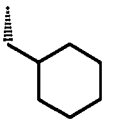
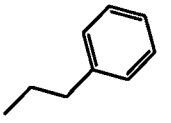
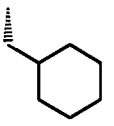
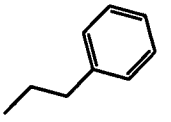
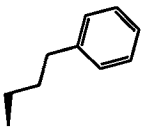
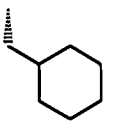
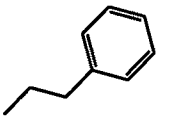
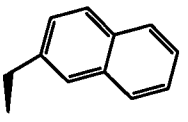
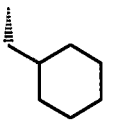
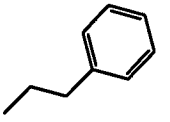
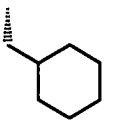
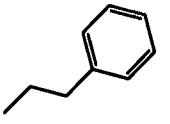
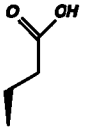
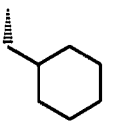
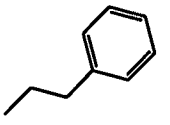
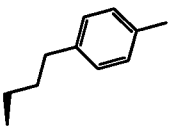
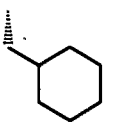
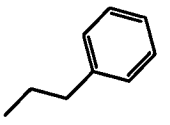
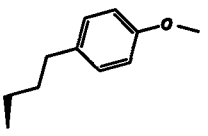
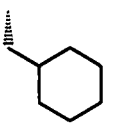
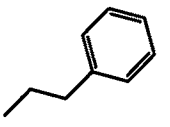
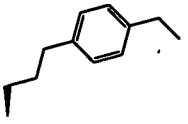
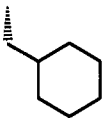
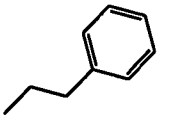
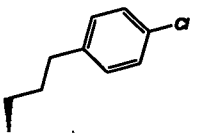
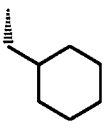
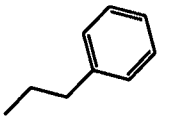
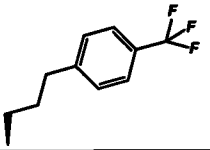
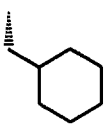
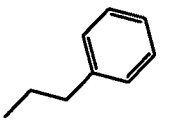
The significant increase in efficiency caused by the presence of the hydroxamate ligand is consistent with the observations of other investigators (reviewed in Johnson *et al.*, 1987). In contrast to the results presented above, highly efficient thiol inhibitors of collagenase have also been described but these invariably have an isobutylsuccinyl group at the P_1' position.

(ii) Modifications of the P_1' position (Table 5.2)

The structures of most current MMP inhibitors have evolved from compounds which were based on amino acid residues bordering the site on collagen which is cleaved by collagenase. The P_1' position of collagen is occupied by leucine or isoleucine and there is evidence that the preference of collagenase for these residues extends to substrate based inhibitors (reviewed in Johnson *et al.*, 1987). Collagen is, however, resistant to cleavage at this position by any other member of this family and indeed coliagenase only hydrolyses this bond with the aid of a non-catalytic element provided by its C-terminal domain. Preliminary studies identified this position as a target for building gelatinase-A selectivity over collagenase. Early SAR data was generated by using stromelysin rather than gelatinase-A but the two enzymes are predicted to possess similar

Table 5.2

Modifications of the P_1' Position of Hydroxamate Inhibitors

CT No	P_1'	P_2'	P_3'	Activity / Ki [nM] GL-A	SL	CL
435				0.3	25.3	7.8
539				0.54	19.1	15.1
466				2.24	77	618
471				0.06	8.3	203
574				200	15,000	30,000
743				8	338	415
763				44	94,000	60,000
921				0.08	8.2	921
1043				0.06	4.2	1,530
1044				0.1	5.4	5,120
1071				0.03	7.3	2,440
1291				0.3	90	5,000

binding pockets based on their substrate specificity. Compounds with a range of modifications at the P_1' were analysed by measuring their IC_{50}'s for stromelysin using a [^{14}C]-casein assay (section 2.2:6). These studies identified the hydroxamic acid inhibitor, CT435 (Table 5.2), as a lead compound. CT435 possesses a cyclohexylalanine at the P_2' position, phenethyl amide at the P_3' position and a residue based on leucine (isobutyl) at its P_1' site. Analysis of this inhibitor using peptide assays showed it to be highly effective against gelatinase-A (Ki = 0.18 - 0.3 nM), 25 fold less potent on collagenase (7.8 nM) and 75 fold less potent on stromelysin (25.3 nM). Extension of the P_1' hydrocarbon chain to C_5 (CT539) had little effect on either affinity or selectivity for gelatinase-A although some activity against collagenase was lost.

A phenethyl group was introduced (CT466) into the P_1' position, based on the observation that the gelatinases and stromelysin hydrolyse peptide bonds on the amino side of aromatic amino acids during auto-catalysis. Surprisingly, affinity for all the enzymes was reduced, although there was a significant increase in selectivity for both gelatinase-A and stromelysin over collagenase. This latter observation is consistent with the substrate data. Shortening the hydrocarbon chain by a single carbon further accentuated this finding (data not shown). Extension of the hydrocarbon chain to produce a phenylpropyl group (CT471) highlighted differences between collagenase and the other enzymes at this position. The affinity for gelatinase-A was significantly improved (0.06 nM) as was that for stromelysin (8.3 nM) but inhibition of collagenase remained relatively poor (203 nM). Interestingly, replacement of phenylpropyl with a phenylbutyl reduced activity on gelatinase-A, thereby, defining the length of the S_1' channel (data not shown).

In order to probe the nature of this interaction additional modifications were evaluated. Substitution of the phenyl group with naphthalene (CT574) resulted in a reduction in activity of greater than 1000 fold for all the enzymes which indicates an upper size limit for the width of this pocket. Incorporation of carboxylate functions based on glutamic acid (CT743 and CT763) was clearly detrimental to inhibitor activity against all test enzymes although the resulting loss in activity was less marked for gelatinase-A which is known to cleave gelatin peptides at glutamic acid (Seltzer *et al.*,1990).

The aryl group is a potential substrate for enzymes of the cytochrome P450 family and therefore a range of substitutions on the aryl ring was tested. In

general, substitutions at sites other than the para position were detrimental to activity on gelatinase-A (data not shown). Inclusion of a toluyl group (CT921) at this position improved selectivity over collagenase. In later inhibitor series this modification also resulted in a minor improvement in activity against gelatinase-A (data not shown). Selectivity against collagenase was further enhanced by increasing the size of the group at the para position (CT1043, CT1044 and CT1071). Substitution with chlorine (CT1071) or methoxy (CT1043) groups consistently produced the most highly active inhibitors in each series whereas no pattern emerged for other groups such as fluorine (CT1291). Modifications along the hydrocarbon chain were examined using the CT1746 series. Interruption of the chain with oxygen was tolerated to some extent by gelatinase-A, whereas, the introduction of branching lead to a reduction in activity dependent on the number and position of the additional methyl groups (data not shown).

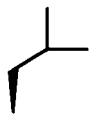
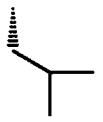
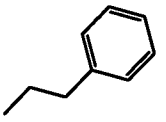
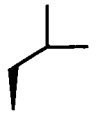
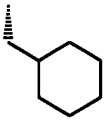
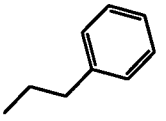
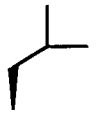
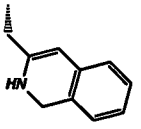
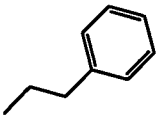
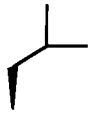
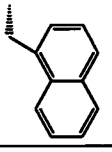
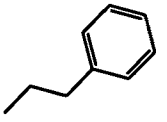
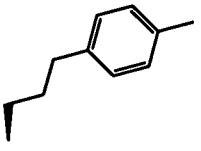
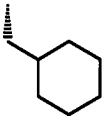
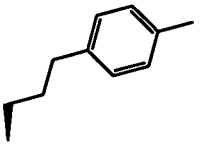
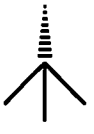
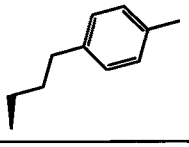
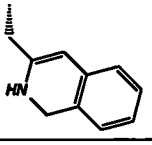
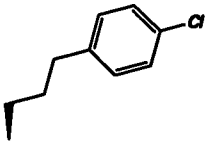
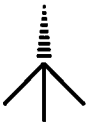
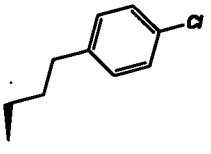
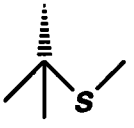
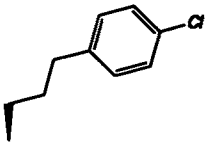
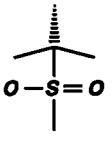
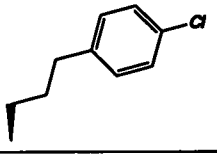
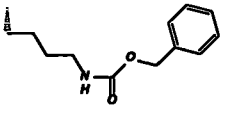
The intolerance of collagenase to extensions of the P_1' chain and in particular to the addition of an aryl group is evidence that the pocket is closed off. The gelatinases and stromelysin on the other hand facilitate such a group and, moreover, the improved activity of these inhibitors indicates the presence of additional interactions of the aryl group with the enzyme's binding pocket. These enzymes may be visualised as possessing a large hydrophobic space beyond the S_1' channel which may be present in collagenase but is inaccessible to inhibitors. Activity against gelatinase-A is consistently 50 - 100 fold higher than against stromelysin although the SAR for the two enzymes is similar suggesting that they possess similarly shaped substrate binding pockets.

(iii) Modifications of the P_2' position (Table 5.3)

The P_2' position was first explored by measuring Ki's for a range of compounds which were based on CT420 which has the (S)- stereoisomer of isobutyl at this site. Replacement of this group by the leucine mimic, cyclohexylalanine (CT435) did not effect activity on collagenase but resulted in a 10 fold increase in affinity for gelatinase-A and a 2 fold increase for stromelysin. The presence of this group conferred both improved activity and selectivity for gelatinase-A and formed the basis for the design of highly active, selective

Table 5.3

Modifications of the $P_{2'}$ Position of Hydroxamate Inhibitors

CT No	P_1'	P_2'	P_3'	Activity / Ki [nM] GL-A	SL	CL	GL-B
420				2.85	45	7.6	–
435				0.33	25.3	7.8	–
455				0.36	55	18.6	–
493				0.73	20.2	5.5	–
1408			CH_3	0.01	3.0	302	0.03
1622			CH_3	0.02	9.91	84.4	0.14
1670			CH_3	<0.01	2.17	35.8	0.07
1694			CH_3	0.01	2.64	22.5	0.1
1711			CH_3	<0.01	5.3	26.3	0.05
1713			CH_3	<0.01	5.92	20.7	0.05
1727			CH_3	0.01	6.1	233	0.16

inhibitors of this enzyme. A variety of alternative groups were tested. Incorporation of a tryptophan at this position has been shown by several groups to be potency enhancing for collagenase (Kortylewicz *et al.*, 1990) but this did not prove to be the case in the CT420 series (CT455). Affinity for collagenase was slightly reduced whereas affinities for gelatinase-A and stromelysin were similar to those produced by cyclohexylalanine. Inclusion of a naphthalene group at this position (CT493) reversed this process by restoring activity against collagenase and reducing activity on gelatinase-A and stromelysin. The effect of cyclohexylalanine replacement was also examined in the CT1408 and 1694 series. Replacement by a tertiary-butylglycine (BUG) caused a slight reduction in gelatinase-A, gelatinase-B and stromelysin potency whilst activity against collagenase was significantly enhanced (CT1622). In this series tryptophan (CT1670) improved affinity for gelatinase-A, stromelysin and also collagenase. The introduction of penicillamine based analogues of tertiary butylglycine to the CT1694 series produced a slight improvement in affinity for both gelatinases, reduced affinity for stromelysin and little effect on collagenase (CT1711, CT1713). Addition of a large P_2' side-chain to produce CT1727 was well tolerated by all the enzymes except for collagenase.

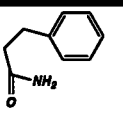
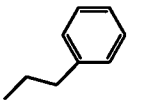
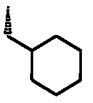
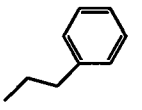
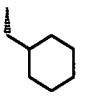
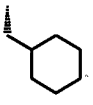
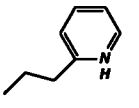
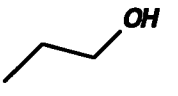
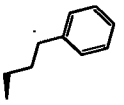
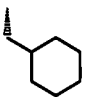
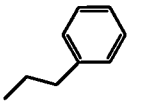
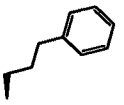
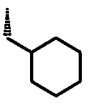
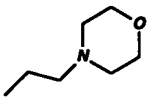
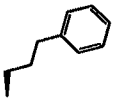
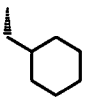
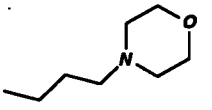
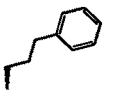
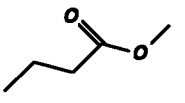
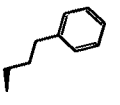
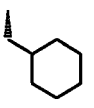
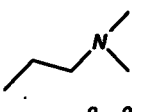
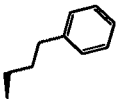
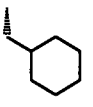
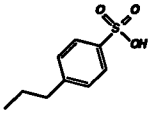
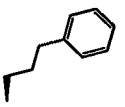
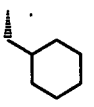
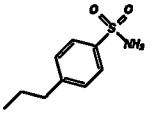
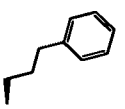
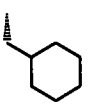
A broad range of groups is tolerated at the P_2' position. Both the size and variety of side-chains accepted at this site indicate that the corresponding enzyme pocket is orientated away from the body of the enzyme and towards the solvent.

(iv) Modifications of the P_3' position (Table 5.4)

Early work on the P_3' site focused primarily on modifications of CT383, which had an isobutyl group occupying the P_1' position. Removal of the C-terminal amide from CT383 produced compound CT420. This modification, which removes a chiral centre, resulted in a significant improvement in inhibitory activity against collagenase and gelatinase-A and a more modest enhancement of stromelysin inhibition. The phenethylamide group was then incorporated into the active CT435 series which have cyclohexylalanine at the P_2' site. Complete removal of the P_3' group (CT478) was detrimental to activity against all the enzymes in particular stromelysin and collagenase. The phenethylamide group could, however, be replaced with a pyridyl moiety attached to an equivalent hydrocarbon chain (CT480), without affecting the affinity for any of the enzymes.

Table 5.4

Modifications of the P_3' Position of Hydroxamate Inhibitors

CT No	P_1'	P_2'	P_3'	Activity / Ki [nM] GL-A	SL	CL
383				33	129	303
420				2.8	45	7.6
435				0.33	25.3	7.8
478			—*H*	6.32	902	600
480				0.33	27	11
505				327	15.000	7,400
471				0.06	8.3	203
543				0.1	24.8	210
962				0.04	9.4	535
546				0.06	17.7	225
550				0.11	38	393
570				0.59	81.5	942
572				0.02	5.92	150
715			CH_3	0.08	34.5	231
733				2.82	1,020	16,700

Substitution of this group by a hydroxyl (CT505), produced a 50 - 100 fold loss in activity against all the enzymes. The reduction in activity was significantly greater than that resulting from complete removal of this group (CT478) and may indicate that the presence of the hydroxyl distorts the inhibitor structure. The intolerance of the enzymes to P_3' hydroxyl groups is confirmed by studies conducted on later series of compounds (data not shown). A broad selection of P_3' modifications was tested in the CT471 series of compounds which all possess a phenylpropyl group at the P_1' position. It is evident from these studies that a range of side-chains of varying charge and size are tolerated at this position by all the enzymes. Examples include CT543 (morpholino), CT546 (ester), CT550 (dimethylamino), CT572 (arylsulphonamide) and CT715 (N-methylamide). An exception to this is provided by the arylsulphonic acid of CT570 which causes some reduction in activity against all the enzymes. Comparison of CT962 with CT543 shows that extension of the hydrocarbon chain by a single carbon can improve activity against gelatinase-A and stromelysin and also improve selectivity over collagenase.

The large variety of groups tolerated at this position, by gelatinase-A in particular, is exemplified by CT733 which has a biotin label attached to an extended hydrocarbon chain. Despite the large size of this group the Ki value for gelatinase-A was relatively low (2.8 nM). Indeed further extension of the chain produces an inhibitor with a Ki of 0.08nM against gelatinase-A (data not shown).

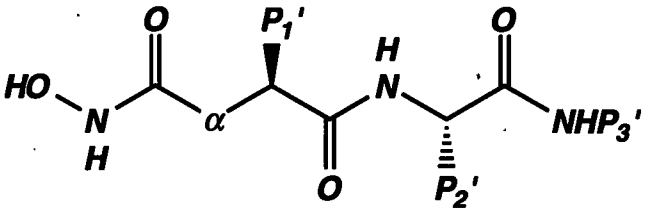
The results indicate that, like the S_2' subsite the S_3' is orientated towards the solvent. For this reason a range of large charged and uncharged groups are tolerated by the enzymes.

(v) Inhibitor selectivity for a range of zinc metalloproteinases (Tabie 5.5)

A wide variety of zinc metalloproteinases are present in the body in addition to the MMP family. Many of these enzymes are membrane-bound peptidases such as aminopeptidase N (AP-N) and the carboxypeptidase, endopeptidase-dipeptidyl A (ACE). Their location on exposed surfaces such as the microvilli of the gut and kidney, endothelial cells and the plasma membrane of lymphocytes make them easily accessible to orally ingested and circulating xenobiotics. Representative compounds (Fig. 5.1) were tested against a range of zinc metalloproteinases which included gelatinase-A, gelatinase-B, stromelysin, collagenase, matrilysin, ACE and AP-N. MMP assays were performed

Figure 5.1

Structures of Zinc Metalloproteinase Inhibitors



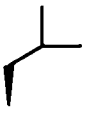
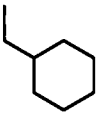
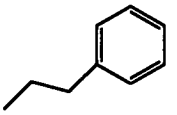
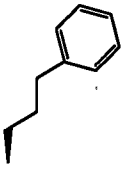
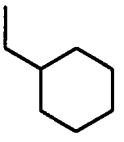
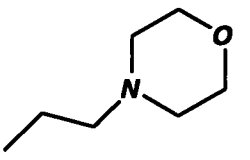
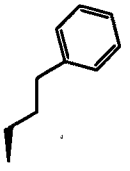
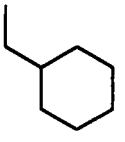
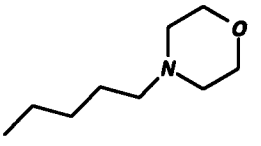
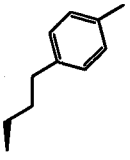
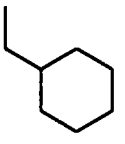
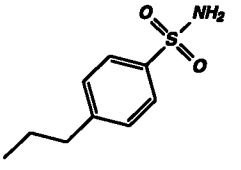
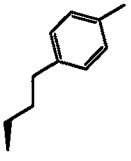
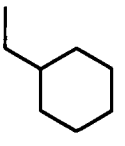
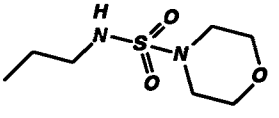
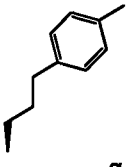
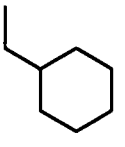
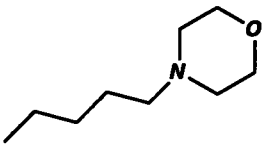
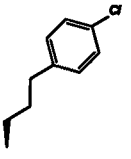
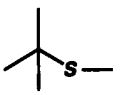
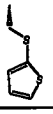
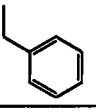
CT No	α	P_1'	P_2'	P_3'
435	H			
543	H			
962	H			
989	H			
1166	H			
1509	H			
1746	H			H
1847	H			CH_3
Ro31-9790	H			CH_3
BB94				CH_3

Table 5.5

Inhibition of Zinc Metalloproteinases

	ENZYME						
INHIBITOR	*GL-A*	*GL-B*	*SL*	*CL*	*PUMP*	*ACE*	*AP-N*
CT435	0.33	3.6[a]	25.3	7.8	14.0	>100,000[b]	<1000[c]
CT543	0.14	2.1[a]	24.8	210	ND	ND	ND
CT962	0.04	1.1[a]	9.4	535	ND	ND	ND
CT989	0.01	0.08[a]	3.0	329	ND	ND	ND
CT1166	0.01	0.04[a]	2.8	385	6400	ND	ND
CT1509	0.035	0.47[a]	2.4	313	ND	ND	15,000[c]
CT1746	0.04	0.42[a]	10.9	122	136	>100,000[b]	83,000[c]
CT1847	1.55	5.2[a]	90	2.9	5.0	>100,000[b]	13,000[c]
Ro31-9790	1.8	6.0[a]	119	4.2	4.9	>100,000[b]	12,000[c]
BB94	0.26	0.47[a]	2.0	1.5	1.4	>100,000[b]	ND

Numbers represent Ki's (nM) measured against DnpPLGLWAR except;

(a) represents Ki_{app} (nM) measured against DnpPLGLWAR

(b) represents an IC50 (nM) measured against furylacryloyl - F - G - G (328 nm)

(c) respresents an IC50 (nM) measured against L - Leucine p - nitroanilide (405 nm)

GL-A = Gelatinase-A
GL-B = Gelatinase-B
SL = Stromelysin
CL = Collagenase

PUMP = Punctuated Metalloproteinase (Matrilysin)
ACE = Peptidyl-dipeptidase A
AP–N = Aminopeptidase–N
ND = not done

using DnpPLGLWAR. ACE activity was monitored continuously using the furylacryloyl - F -F- G substrate (λ_{328}) and AP-N activity was monitored using the substrate L-Leucine-p-nitroanilide (λ_{405}). The results of these studies are presented in Table 5.5

None of the inhibitors exhibited any significant activity against the carboxypeptidase, ACE. This result is consistent with the requirement of carboxypeptidases for a free C-terminal carboxylate on their substrates.

Compounds with isobutyl occupying the P_1' site (CT435, CT1847, Ro31-9790) were more potent inhibitors of AP-N than those with arylpropyl groups at this position. CT435 exhibited an IC_{50} of less than 1μM for AP-N which was comparable to that of actinonin, a natural inhibitor isolated from actinomycetes (data not shown). This result is compatible with the observation that the two inhibitors possess common structural features (Fig. 1.8). CT1847 and Ro31-9790 do not inhibit AP-N as efficiently as CT435 despite the presence of a conserved P_1' group. This may reflect a preference of AP-N for a bulky P_3' residue.

Matrilysin does not tolerate an aryl group at the P_1' position. The most active matrilysin inhibitors CT435, CT1847, Ro31-9790 and BB94 all have an isobutyl group at this site. A range of P_1' arylpropyl inhibitors represented by CT1166 all possess micromolar Ki's against matrilysin. An exception to this finding is provided by CT1746 which has a Ki of 136 nM. This indicates that features in addition to the P_1' site are also responsible for determining activity on matrilysin.

The Ki values for these inhibitors against collagenase, stromelysin, gelatinase-B and gelatinase-A confirm and extend earlier observations. A leucine-like P_1' site is an essential requirement for efficient inhibition (low nanomolar) of collagenase (CT435, CT1847, Ro31-9790 and BB94). A range of substituents is tolerated at the P_2' position although groups larger in size than the amino acid valine are generally preferred (data not shown). Collagenase prefers small groups such as N-methylamide at the P_3' which distinguishes it from the gelatinases and stromelysin. The most active collagenase inhibitors, CT1847, Ro31-9790 and BB94 all possess such a group. CT435 on the other hand possesses a phenethylamide in this position. This causes a reduction in activity compared to CT1847 but the flexibility of this group and the absence of a chiral centre may contribute to a significantly better interaction with the enzyme than

that provided by the phenylalanine amide of the parent compound, CT383 (Table 5.4).

As a general rule affinity for stromelysin follows a similar SAR to that observed for gelatinase-A and gelatinase-B. The inhibitors, CT1746 and CT962 for example, differ at all positions but exhibit very similar SARs for all the test enzymes. Whilst selectivity data indicates that these three enzymes have very similar S_1', S_2' and S_3' binding pockets, absolute inhibitor potency varies in the order; gelatinase-A (5 - 10 fold) > gelatinase-B (20 fold) > stromelysin.

The British Biotechnology inhibitor BB94 is interesting in that it possesses a thioether linked group at the α-carbon position. The α-carbon substituent appears to promote a significant enhancement of potency against all the MMPs tested. Despite the absence of a P_1' arylpropyl group, it is highly active against the gelatinases and stromelysin and the compound is unusual in that the relative activities against gelatinase-A and gelatinase-B are reversed. Additionally, the thioether moiety significantly improves activity against collagenase and matrilysin relative to structurally similar inhibitors lacking this group.

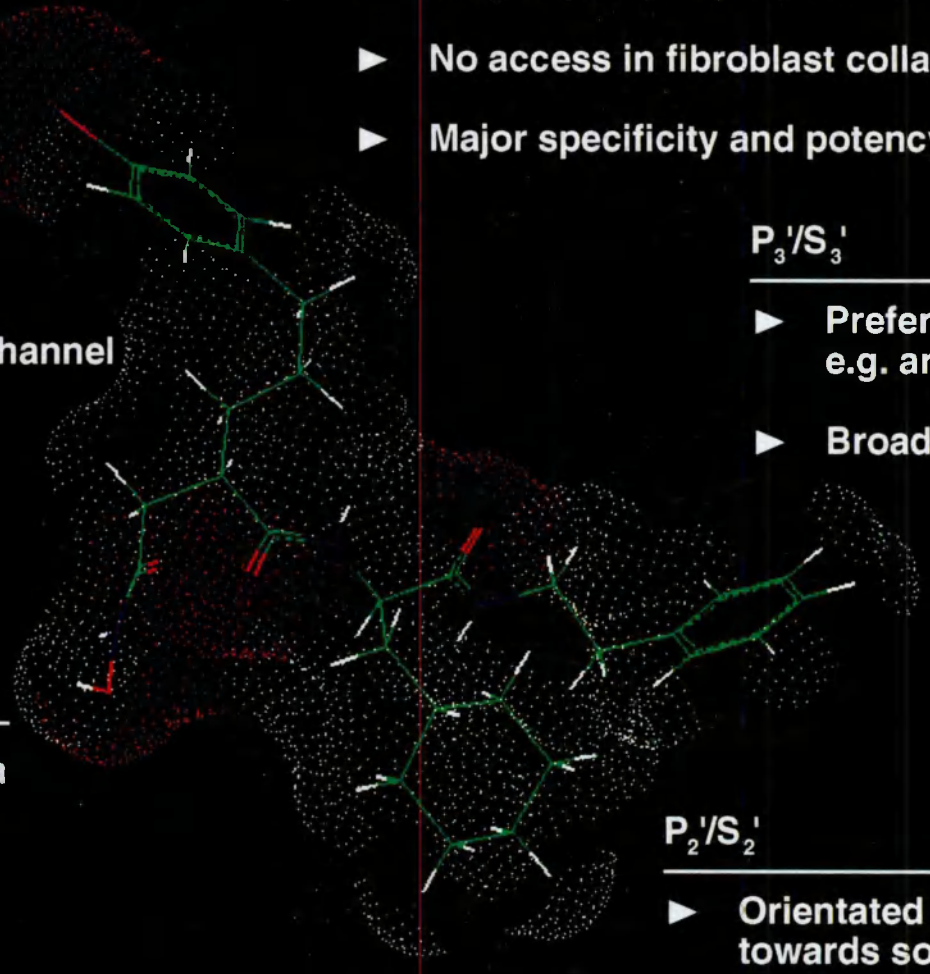
The structure-activity relationship for inhibition of MMPs by hydroxamates is summarised in Fig. 5.2 (A). The model is based on the crystal structure of a related MMP inhibitor and, therefore, the orientation of the side-chains is probably accurate. The corresponding CPK model is presented in Fig. 5.2 (B).

In summary, the presence of phenylpropyl at the P_1' confers selectivity and potency for gelatinases-A and B and stromelysin and is not tolerated either by collagenase and matrilysin or by other zinc metalloproteinases, including AP-N. The S_2' sub-site of the enzyme appears to be orientated towards the solvent and can tolerate a large variety of inhibitor side-chains. A range of substituents is tolerated at the P_3' site although the increased activity observed on extension of the alkyl chain may be a result of interactions away from the S_3' pocket. Whilst the most potent gelatinase-A inhibitors such as CT989 and CT1166 possess very large groups at this position, collagenase appears to prefer a much smaller substituent such as N-methylamide. This class of inhibitors is not recognised by the carboxypeptidase, ACE.

Figure 5.2

A

The structure of a gelatinase inhibitor



Narrow hydrophobic channel

HYDROXAMIC ACID

Bidentate binding to zinc and a hydrogen bond to a backbone carbonyl group

P_1'/S_1'

- Large hydrophobic pocket with preference for aryls
- No access in fibroblast collagenase
- Major specificity and potency pocket

P_3'/S_3'

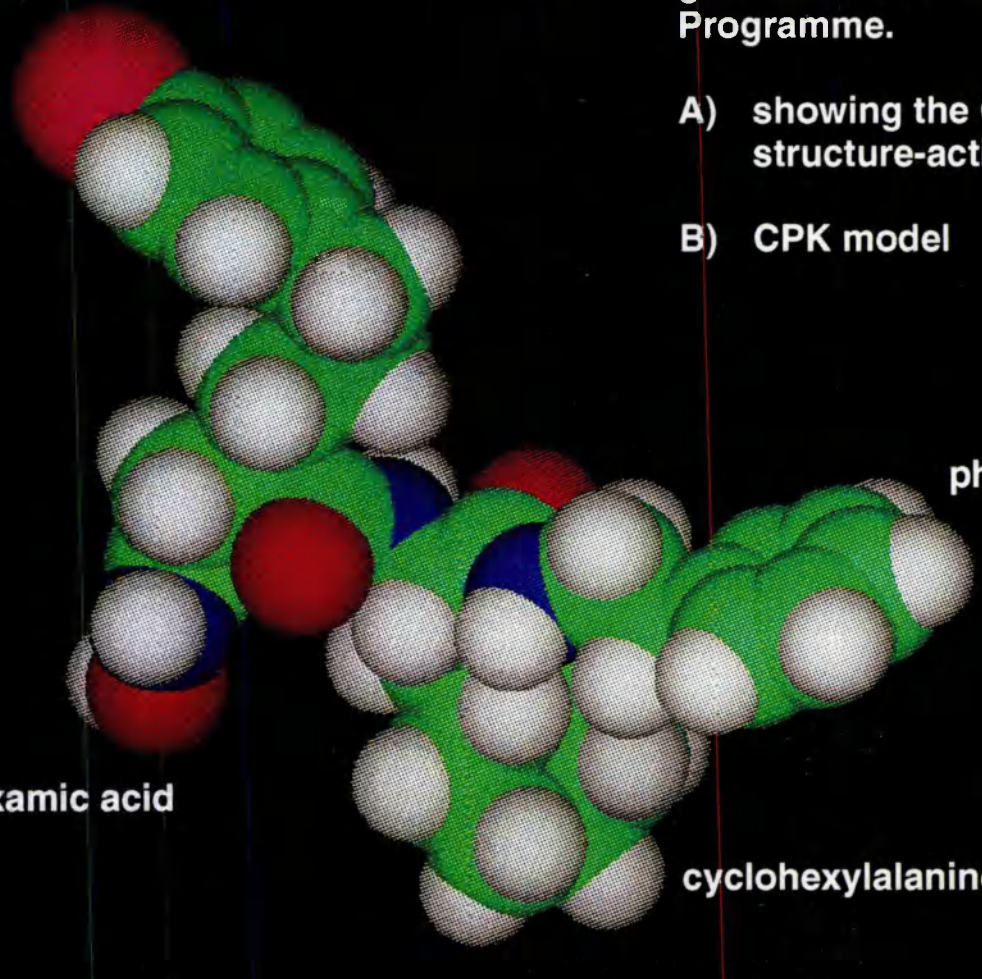
- Preference for bulky groups e.g. aryls
- Broad specificity

P_2'/S_2'

- Orientated away from enzyme towards solvent
- Few specific interactions

Figure 5.2

B



Models are based on the X-ray crystal structure of a gelatinase inhibitor using the Sybyl–Tripos Associates Programme.

A) showing the Connolly surface summarises the structure-activity relationship for gelatinase-A

B) CPK model

5.3: Discussion

Inhibition constants (Ki's) were determined against gelatinase-A, stromelysin and interstitial collagenase for a range of inhibitors of the general structure Z-P_1'-P_2'-P_3' where Z represents a zinc binding ligand (generally hydroxamic acid) and P_1' - P_3' align the inhibitor with corresponding substrate binding sites (S_1' - S_3') on the enzyme. Additional data was also presented for the inhibition of gelatinase-B, matrilysin, aminopeptidase-N and peptidyl-dipeptidase A. SARs were used to define the optimum chemical groups responsible for activity and selectivity for gelatinase-A.

The results of these studies will be discussed in the light of new data provided by the 3-D structures of several MMPs which have been determined by crystallography (Lovejoy *et al.*, 1994a;1994b; Bode *et al.*, 1994; Borkakoti *et al.*, 1994; Stams *et al.*, 1994) and NMR studies (Gooley *et al.*, 1994).

Zinc binding ligand

This study shows that inhibitors with zinc binding ligands (hydroxamic acid and carboxylic acid) capable of bidentate binding were considerably more active against gelatinase-A than inhibitors with monodentate ligands (sulphydryl). Furthermore, hydroxamates were at least 100 fold more active than the corresponding carboxylate for all the test enzymes. This indicates that hydroxamic acid forms an additional bond with the enzyme and from our knowledge of the consequences of modifying the NH group (data not shown) this was presumed to be the point of interaction. This has recently been confirmed by Borkakoti *et al.* (1994) who, having co-crystallised interstitial collagenase with a hydroxamate inhibitor, found that the NH had the potential to hydrogen bond to the carbonyl of an alanine eleven residues downstream of the zinc box. This interaction may be responsible for the well documented potency of hydroxamic inhibitors against MMPs.

P_1' modifications

Requirements for effective S_1' binding were identified by using SAR data provided by a series of inhibitors with invariant P_2' and P_3' side-chains (Table 4.2). Leucine-like groups were tolerated by all the test enzymes but replacement by an aryl group produced a significant improvement in the selectivity and

efficiency of hydroxamate inhibitors for the gelatinases and stromelysin over collagenase and matrilysin. This result is consistent with the ability of the gelatinases and stromelysin to cleave both peptides and macromolecules on the N-terminal side of phenylalanine. Interstitial collagenase, on the other hand, cleaves only at leucine or isoleucine on collagen. Surprisingly, introduction of a side-chain which resembled phenylalanine (phenethyl) reduced activity (CT466) against gelatinase-A and stromelysin but the substrate data was borne out when the chain was extended. Propyl was the optimal hydrocarbon chain for both the gelatinases and stromelysin. The results of SAR analysis indicate that the enzymes possess a narrow hydrophobic channel at this site. The pocket broadens to accept an aryl group in the gelatinases and stromelysin but is blocked in collagenase and matrilysin. This conclusion can be rationalised by comparing the primary amino acid sequence of each enzyme with the published 3-D structures. In all the enzymes, apart from interstitial collagenase and matrilysin, a leucine four residues down-stream of the zinc box enters the S_1' pocket but is directed away from the zinc. In interstitial collagenase this residue is replaced by the larger arginine residue which effectively closes off the pocket. Matrilysin has a tyrosine in this position which may partially block the pocket (see Fig.1.11).

P_2' modifications

The P_2' position may be occupied by a variety of groups of differing size and charge. The first significant progress was made when leucine (CT420) was replaced by cyclohexylalanine, a leucine mimic used extensively in the renin inhibitor area. As a result of this modification, activity against gelatinase-A was increased by an order of magnitude, there was a more modest improvement against stromelysin and collagenase was unaffected. This group, therefore, satisfied the criteria of activity and selectivity for gelatinase-A and became a consistent element in the structure of future potent *in vitro* inhibitors. As a rule groups smaller than leucine were less effective but no upper size limit was apparent. In later studies the tolerance of gelatinase-A to modifications at this subsite provided scope for altering the physiochemical properties of the molecule (eg. CT1694). These studies show that the P_2' side-chain is orientated away from the bulk of the enzyme towards the solvent. A limited degree of potency and selectivity was achieved at this position but beneficial modifications were difficult

to predict from SARs alone. SAR data from other groups substantiates these results (Chapman *et al.*, 1993). Fig. 1.11 shows that a shallow subsite is formed by the side-chain of a single non-conserved residue, thereby confirming the SAR results.

P_3' modifications

The first significant improvement in inhibitory activity against all the test enzymes was discovered upon removal of the C-terminal amide from P_3' phenylalanine (CT383). The resulting phenethylamide (CT420) enhanced inhibitor efficiency by an order of magnitude for gelatinase-A and collagenase with a more modest improvement for stromelysin. This position was identified as a potential site for modifying the water solubility of the inhibitors. Accordingly. an extensive range of substituents were synthesised including, amides, N-methyamides, carboxylic acids, morpholino's, hydroxyls, amines, sulphamylureas and arylsulphonamides. Amongst these only hydroxyls were not tolerated (CT505). Sulphamylurea (CT1166) and arylsulphonamide (CT989) are the most active inhibitors of gelatinase-A described to date. Both possess Ki's significantly below 10^{-11} M and indeed the Ki of CT1166 has been estimated to be 10^{-12} M. The observation that biotinylated molecules were recognised by the biotin specific agent streptavidin indicates that the P_3' resides at or near the surface of gelatinase-A.

Although collagenase can accept large extended groups at this position most active inhibitors have a small substituent such as methylamide. This is not unexpected as the corresponding residue in collagen is glycine.

There are two possible explanations for the results described above.

1/ The S_3' subsite is similar to the S_2' pocket in being orientated towards the solvent. The wide variety of side-chains tolerated by the enzyme at this position provide some evidence for this.

2/ The S_3' pocket is small and hydrophobic as indicated by SARs for collagenase. Extension of the hydrocarbon chain which was a feature of highly active inhibitors may effectively by-pass the S_3' subsite.

NMR studies on the catalytic domain of neutrophil collagenase confirm that the first hypothesis is correct (Stams *et al.*,1994).

Selectivity for MMPs

Lack of selectivity may result in toxicity and there are a range of related zinc-dependent metailoproteinases in the body. Accordingly, a selection of hydroxamic acid inhibitors were tested against an aminopeptidase (AP-N) and a carboxpeptidase (ACE) in addition to five MMPs. None of the test compounds showed any inhibitory activity against ACE. This is not unexpected as the specificity of this enzyme for the C-terminus of peptides is determined by its interaction with the free carboxyiate group of the substrate. It does not hydrolyse C-terminally blocked peptides for instance. Surprisingly, compounds with P_1' aryl showed relatively poor activity against AP-N aithough the enzyme hydroiyses substrates with a corresponding phenylalanine. Potent inhibitors of such peptidases do have a phenylalanine at this position which indicates that the lack of inhibitory activity is a consequence of extending the hydrocarbon chain. In this study compounds with a P_1' aliphatic group (CT435) inhibited AP-N with IC_{50}'s in the nanomoiar range. Comparison of the results for CT435, CT1847 and Ro31-9790 indicate the presence of a significant P_3' interaction. A similar inhibitor profiie has been reported for neprilysin and mouse meprin, a non-MMP member of the metzincin famiiy (Dr. A. Kenny, personai communication).

BB94 is unique amongst these inhibitors in possessing an α-carbon substituent. Its presence in the (S)- form augmented the activity of corresponding isobutyl inhibitors against a range of MMPs. This might arise from it extending into the S_1' space which is occupied by an aryl ring in gelatinase inhibitors. This idea is not consistent, however, with molecular modeliing studies based on collagen (Johnson *et al.*,1987) which indicate that groups at this position point away from the S_1' pocket. This class of inhibitor is yet to be co-crystaiiised with an MMP and so the role of the α-carbon side-chain remains unclear.

In summary, selective, picomolar inhibitors of geiatinase-A have been deveioped with the aid of SAR data. The hydroxamic acid zinc binding iigand and the alignment of the pseudo-peptide side-chains with the substrate recognition sites on the enzyme both contribute to the high strength of binding. On the enzymes, the S_1' pocket is the major site of substrate / inhibitor recognition and therefore is the primary target for obtaining potency and selectivity. Other prime-side pockets are shallow which allows scope for manipuiating the physicochemicai nature of inhibitors to provide improved *in vivo* characteristics.

The construction of SARs on isoiated enzymes are only one aspect of inhibitor design. In Chapter 7 the relationship of structure to *in vivo* activity wili be investigated.

Chapter 6

CHAPTER 6

KINETIC ANALYSIS OF INHIBITOR BINDING

6.1: Introduction

Chapter 5 described how SARs can be used to discover potent, selective inhibitors. The data was presented largely in the form of Ki's which presumes reversibility. Information derived from Ki's may be extended by analysing the components of this term, namely the rates of association and dissociation of the enzyme-inhibitor complex. The following study examines mechanisms of MMP inhibition and the nature of the enzyme-inhibitor interaction.

The relationship between the experimentally determined apparent Ki of a reversible inhibitor and the true Ki varies according to whether the inhibitor is competitive with substrate, uncompetitive or non competitive. The use of robust soluble peptide assays allows these properties to be determined. Conversely, information on the behaviour of inhibitors on solubie substrates *in vitro* may not necessarily extrapolate to the *in vivo* situation. Selected inhibitors were, therefore, also tested for their ability to inhibit gelatinase-A in a more natural environment provided by gelatin, a physiological substrate.

Inhibitors of MMPs possess many of the characteristics of the ACE and neprilysin inhibitors including Ki's in the range of 10^{-8} - 10^{-12} M. As a consequence of this high affinity these inhibitors exhibit tight-binding inhibition rather than classical reversible inhibition (section 1.4:5). Since the inhibitor concentration must be varied around its Ki during any inhibition experiment it is likely that inhibitor concentrations will be equivalent to that of the enzyme. Accordingly, the assumption that the free inhibitor concentration is equal to the total inhibitor concentration is not valid and therefore a steady-state treatment of the results is inadequate. A general steady-state rate equation that ailows for the mutual depletion of inhibitor and enzyme was derived by Morrison (1969). The theory was further extended by Cha (1975, 1976) who considered the situation where the rate of establishment of equiiibrium between free enzyme, inhibitor and enzyme-inhibitor complex is slow relative to the rate of substrate conversion.

Apparent Ki's (Ki_{app}) may be determined for such inhibitors using equilibrium methods by preincubating the inhibitor and enzyme solution untii equilibrium has been established. The reaction may then be started by the

addition of substrate. The Ki_{app} can be determined by a non-linear regression of the equation for tight-binding inhibition described by Morrison and Walsh (1988) (section 2.2:4). If the substrate concentration is significantly lower than the K_m then competition for the enzyme is negligible and the Ki_{app} approximates to the true Ki.

Additional information can be obtained from studies of the pre-steady-state kinetics. The slow establishment of equilibria between enzyme, inhibitor and enzyme-inhibitor complexes leads to time-dependent inhibition. Such 'slow-binding inhibition' commonly arises from a slow off-rate (k_{off}) whilst the on-rate (k_{on}) may be fast or slow. This form of inhibition is a common property of inhibitors with low Ki's In these circumstances when the inhibitor concentration [I] is varied in the region of its Ki, values for the pseudo first order rate constant for the formation of the enzyme-inhibitor complex (k_1I) (see page 36) and the rate constant for the dissociation of the compiex (k_2) would be low. The low association and dissociation rates would result in slow-binding inhibition even though the former is of a magnitude expected for a diffusion limited reaction.

Mechanisms to account for slow-binding inhibition were discussed in Chapter 1 (section 1.4:5). Essentially, the two simplest mechanisms to account for any complex formation are mechanism (A) simple bimolecular collision or (B) a pathway that includes intermediates. They may be distinguished by the dependence in mechanism B of the initial steady-state velocity (v_o) on [I]. Additionally, k_{obs} (a pseudo-first order rate constant, equation **{4}**) exhibits saturation at high inhibitor concentration. in practice, the two mechanisms may be difficult to distinguish. Firstly, large errors may be introduced into the measurement of v_o by excessive reactant mixing times. This is particularly evident at the comparatively high inhibitor concentrations required to determine the Ki relating to the initial 'collision' complex. Conversely, it is apparent from the general equation for classical linear competitive inhibition (page 35) that the term $[I]/_{Ki}$ becomes insignificant when [I] << Ki in which case v_o is invariant and mechanism B degenerates to mechanism A.

The following pre-steady-state analyses were performed under the limitations described above which favour mechanism A although preliminary studies on the hydroxamate inhibitor CT435 showed that v_o was not significantly reduced in the presence of increasing concentrations of inhibitor. Analyses of the pre-steady-state kinetics of several inhibitors were performed on the progress

curves obtained by incubating a range of inhibitor concentrations with a fixed concentration of enzyme. These results are then discussed with reference to the contribution of the inhibitor side-chains to the rates of association and dissociation with gelatinase-A.

The studies described above do not provide information on how gelatinase-A binds to a hydroxamic acid inhibitor. It is possible, for example, that the nitrogen atom of hydroxamic acid binds to the oxygen of the glutamic acid (E^{375}) and that this is responsible for the greater potency of such inhibitors over corresponding carboxylate inhibitors. Recent crystallographic data, however, supports an alternative hypothesis in which the NH forms an interaction with a peptide backbone carbonyl group (Borkakoti *et al.*, 1994; Stams *et al.*, 1994) (see Fig. 1.11). Moreover, the absence of such an interaction has been described for the zinc endopeptidase, neprilysin (Devault *et al.*, 1988). In this study, a catalytically compromised mutant gelatinase-A (gelatinase-A{$E^{375}\rightarrow D$}), was used to examine the contribution of this amino acid to the high affinity of hydroxamate inhibitors.

TIMPs are unable to bind to the catalytic domain of MMPs which have an intact propeptide. Low molecular mass synthetic inhibitors may, however, diffuse into the catalytic site as a consequence of their small size. This would not be a desirable property, since it would lead to removal of inhibitor from the circulation by already inactive proenzyme. Two independent methods were used to analyse this property.

6.2: Results

6.2:1 The kinetics of gelatinase inhibition

(i) Inhibitor Mechanism

Methods for the evaluation of inhibition constants (Ki's) are dependent on the mode of inhibition. Because the compounds are structurally related to the substrate early data were analysed on the assumption that the enzyme-inhibitor interaction was both reversible and competitive. The design of more robust peptide assays has subsequently permitted these parameters to be easily investigated. A knowledge of the basic mechanism of the inhibitor-enzyme interaction is also essential for examining the kinetics of association and dissociation and these studies will be described later.

(a) Reversibility

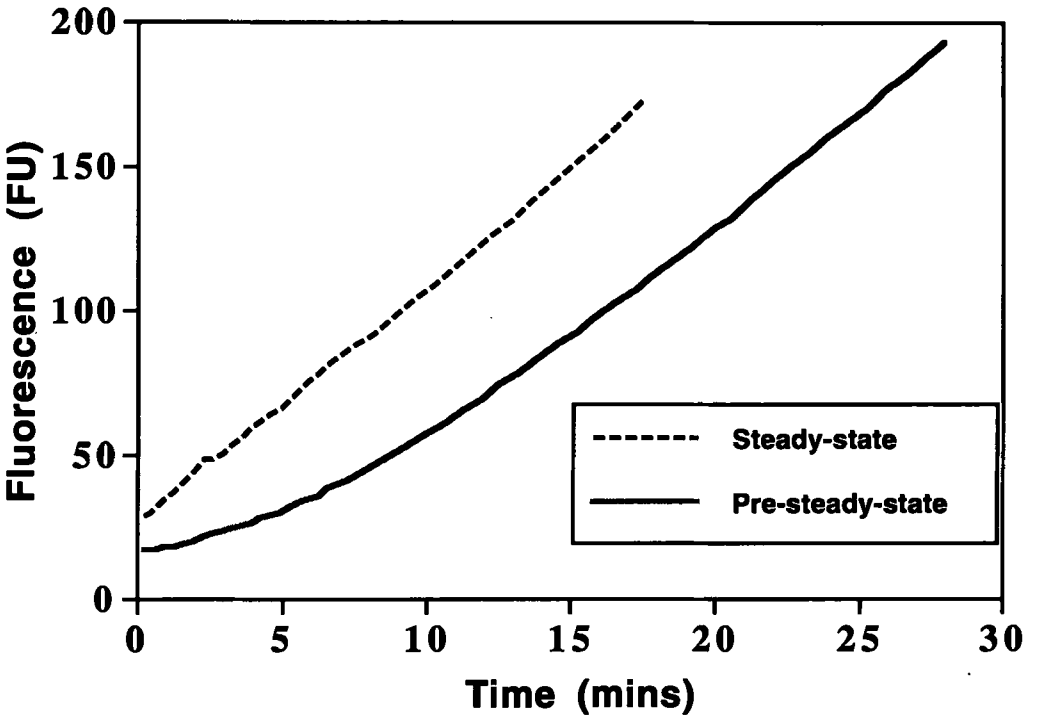
The reversibility of gelatinase-A inhibition by CT1746 was tested as follows. Enzyme-inhibitor solution (0.05 nM) was tested either (A) following a 3 hour preincubation at full assay volume to permit the attainment of equilibrium or (B) following a 3 hour preincubation of a concentrated (5 nM) solution of enzyme and inhibitor. After 3 hours, the QF24 substrate was added to the mixtures to initiate the reaction. Equivalent assays, performed in the absence of inhibitor showed that the attainment of a steady-state rate was distinct and measurable and not due to loss of linearity resulting from substrate depletion, product quenching or enzyme instability.

CT1746 preincubated with gelatinase-A in a full volume of buffer (A) elicited a steady-state rate of 8.3 FU/min (Fig. 6.1). Sample B produced rates of 0.4 FU/min and 8.2 FU/min at 1 minute and 25 minutes, respectively. These results indicate that the interaction between gelatinase-A and CT1746 is fully reversible.

(b) Competitive inhibition of matrix metailoproteinase inhibitors

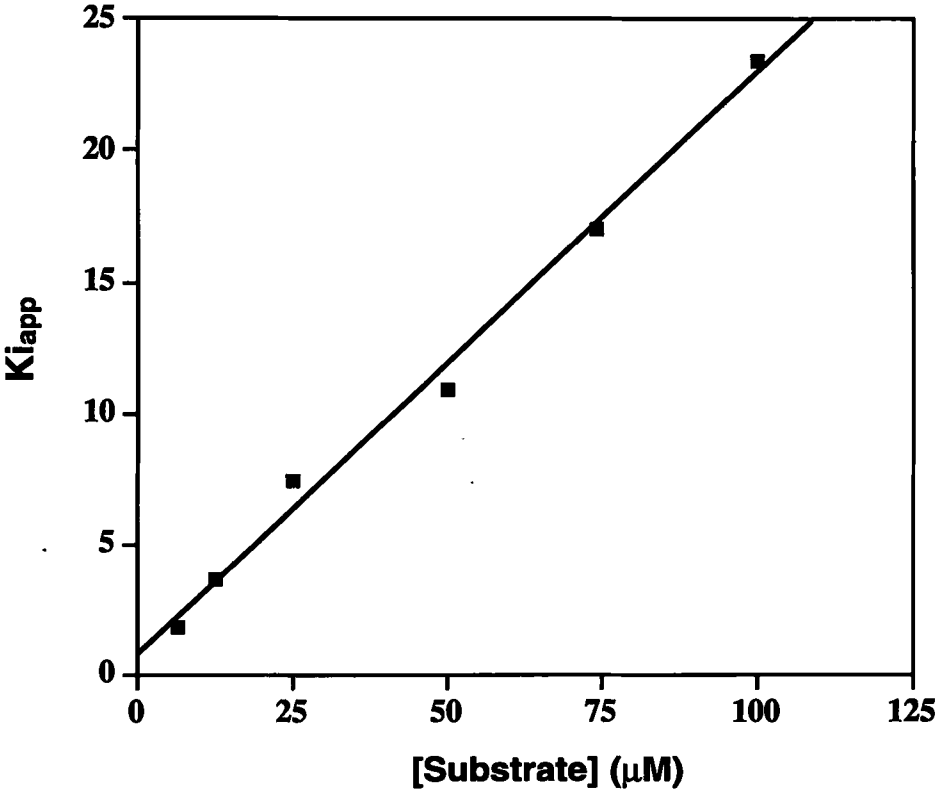
Competitive inhibition is characterised by an increase in Ki_{app} with increasing substrate concentration. From the relationship for competitive inhibition $Ki_{app} = Ki + s.\frac{Ki}{K_m}$ we can see that there is a linear dependence of Ki_{app} and substrate concentration with intercepts of Ki and K_m. Gelatinase-B

Figure 6.1 Reversible inhibition of geiatinase-A by CT1746



CT1746 and gelatinase-A were mixed in peptide assay buffer to a final concentration of each of 5 nM. An aliquot (25 µl) was then removed to 2.465 ml of the same buffer, either immediately (A) or after a 3 hr incubation at room temperature (B). Sample A was incubated for 3 hrs to allow the enzyme and inhibitor to attain equilibrium. Following incubation, 10 µl of QF24 (final concentration, 1.4 µM) was then added to each sample to start the reaction. The rate of substrate hydrolysis was measured as described in Chapter 2.

Figure 6.2 Mode of inhibition of CT1847



Gelatinase-B (0.03 nM) was incubated with a range of Dnp-PLGLWAR concentrations in the presence of 0 - 100 nM of CT1847. Apparent Ki's (Ki_{app}) were determined for each substrate concentration using the equation for tight-binding inhibition (Morrison and Walsh, 1988) (Chapter 2). From the relationship for competitive inhibition $Ki_{app} = Ki + s.\frac{Ki}{K_m}$ a plot of Ki_{app} versus inhibitor concentration will intercept the y-axis at the Ki (0.83 nM) and the x-axis at - K_m (3.9 μM).

was used in preference to gelatinase-A for this purpose since the K_m of the latter for DnpPLGLWAR approaches the limit of solubility of the substrate.

Ki_{app}'s were determined for CT1847 (Fig. 5.1) against gelatinase-B at a range of DnpPLGLWAR concentrations (6.25 - 100 μM). Results were analysed by the tight-binding method of Morrison and Walsh (1988). The resulting plot of Ki_{app} *versus* substrate concentration is presented in Fig. 6.2 It is characteristic of competitive inhibition with intercepts on the y-axis and x-axis, giving values of 0.83 nM and 3.9 μM for the Ki and K_m, respectively.

(ii) Inhibition of gelatinase-A (Table 6.1)

A kinetic study was carried out on selected compounds which represent lead molecules at different stages of the gelatinase inhibitor programme. The structures of these inhibitors are presented in Fig. 5.1. Inhibition of gelatinase-A was studied using several substrates and data were collected from both steady-state analyses and pre-steady-state analyses. IC_{50} values were calculated using gelatin in order to establish that inhibitors would be effective in a more physiological environment. These assays are described in detail in Chapter 2. Approximate Ki values (Ki_{app}) were determined using DnpPLGLWAR at a concentration of 20 μM. Ki_{app} values were converted to Ki's using the relationship for competitive inhibition, $Ki_{app} = Ki\left(1 + S/K_m\right)$ where the K_m for gelatinase-A has been shown to be 70 μM (Chapter 3). All detailed kinetic studies were undertaken using QF24 at a concentration of 1.4 μM, which is significantly below the estimated K_m.

(a) IC_{50} determination

IC_{50} studies using gelatin were limited by the sensitivity of the assay which required a gelatinase-A concentration of 0.5 nM. Under conditions where inhibitor concentration [I] ~ enzyme concentration [E] the following equations describe the influence of the enzyme and substrate concentration on the IC_{50} value for a competitive inhibitor (Morrison and Stone, 1985). Rearrangement of the general equation for tight-binding inhibition (Henderson, 1972) gives,

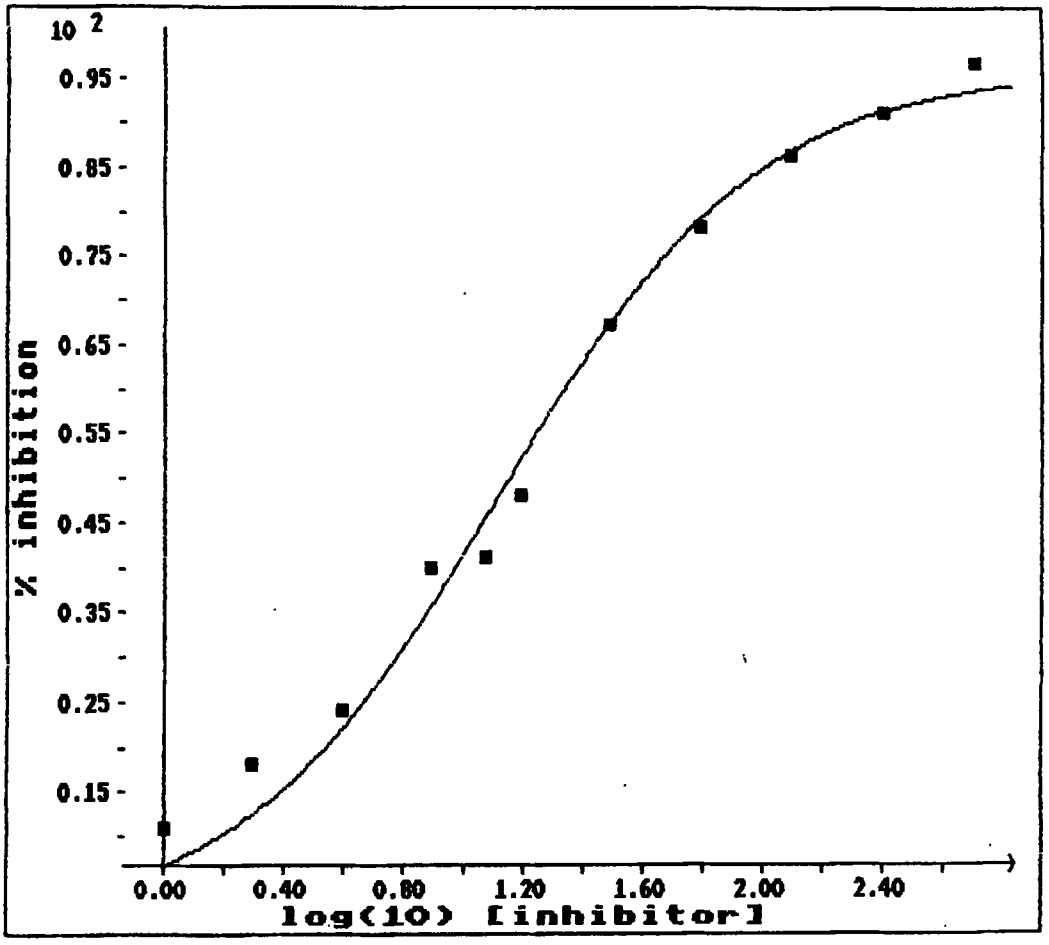
$$\frac{I}{1 - v_i/v_o} = [E] + Ki\left(\frac{s + K_m}{K_m}\right) v_o/v_i \qquad (1)$$

where v_i and v_o are the respective rates in the presence and absence of inhibitor.

Figure 6.3 Inhibition of gelatinase-A action on gelatin by CT976: IC50 Determination

Gelatinase-A (0.5 nM) was incubated with [^{14}C]-gelatin (1 μM) for 100 minutes at 26°C together with CT976 at concentrations ranging from 0 - 1000 nM. Radioactivity remaining in the TCA supernatants (expressed as % inhibition of an enzyme alone control) was then plotted against Log_{10} CT976 concentration. The IC50 value was then determined using Enzfitter (Leatherbarrow, 1987). Each point represents the mean of three determinations.

Figure 6.3 ***Inhibition of Gelatinase-A Action on Geiatin by CT976: IC_{50} Determination***



IC_{50} Determination for CT976 Against Gelatinase-A

IC_{50} Determination
Simple weighting

Variable	Value	Standard Error
IC_{50}	1.11738E+00	4.12102E-02
Limit	9.58972E+01	2.33545E+00

At an inhibitor concentration equivalent to the IC_{50}, $v_i = v_{o/2}$, and this reduces to

$$IC_{50} = {}^{[E]}/_{2} + Ki\left(\frac{s+K_m}{K_m}\right) \quad \textbf{(2)}$$

The concentration of gelatin in this assay approximates to the K_m (1.2 μM) (Table 3.1) and thus,

$$IC_{50} = {}^{[E]}/_{2} + 2Ki \quad \textbf{(3)}$$

and in the majority of cases as Ki << [E] the IC_{50} becomes equivalent to half the enzyme concentration i.e. titrates the enzyme. Accordingly, inhibitors that exhibited Ki's significantly below [E] using peptide substrates, (eg. CT962, CT989, CT1166 and CT1746) had IC_{50} values of < 1 nM. A typical IC_{50} curve for a less active inhibitor, the carboxylate CT976 (IC_{50} = 13 nM), is presented in Fig. 6.2. From the above equation this corresponds to a Ki of 5.7 nM. This value is higher than the Ki determined using peptide substrates (0.45 - 0.9 nM) which suggests that the activity of MMP inhibitors is decreased in the presence of gelatin. Corresponding values for other inhibitors (data not shown) confirm that the Ki is elevated about 10 fold in this assay. The most likely explanation for this finding is that, since gelatinase cleaves gelatin at multiple sites, the true substrate concentration is much higher than the gelatin concentration. Under these circumstances equation **(3)** becomes invalid.

(b) Steady-state analysis on peptide substrates

Ki values were calculated as described in section 2.2:4 by assaying for residual activity after a 3 - 4 hour preincubation of inhibitor and enzyme at 23°C in a full assay volume. The attainment of a steady-state rate was verified for CT435, which has a comparatively slow association rate, by also preincubating enzyme and inhibitor for 16 hours prior to substrate (QF24) addition and comparing the resulting Ki's determined under both sets of conditions. The Ki curve for CT435 after 4 hours preincubation is presented in Fig. 6.4. The Ki value (0.18 nM) calculated from this curve is comparable to that produced after a 16 hour preincubation period (0.25 nM) indicating that 4 hours is sufficient time to attain equilibrium. This study also demonstrates that the enzyme is relatively stable for prolonged periods at low concentration, a property which is a prerequisite for accurate analysis of pre-steady-state kinetics. Table 6.1 shows Ki data for the DnpPLGLWAR substrate and for the QF24 substrate. Comparison of the Ki values generated from the two assays shows the former to be consistently higher which underlines the difficulty in analysing data from assays where

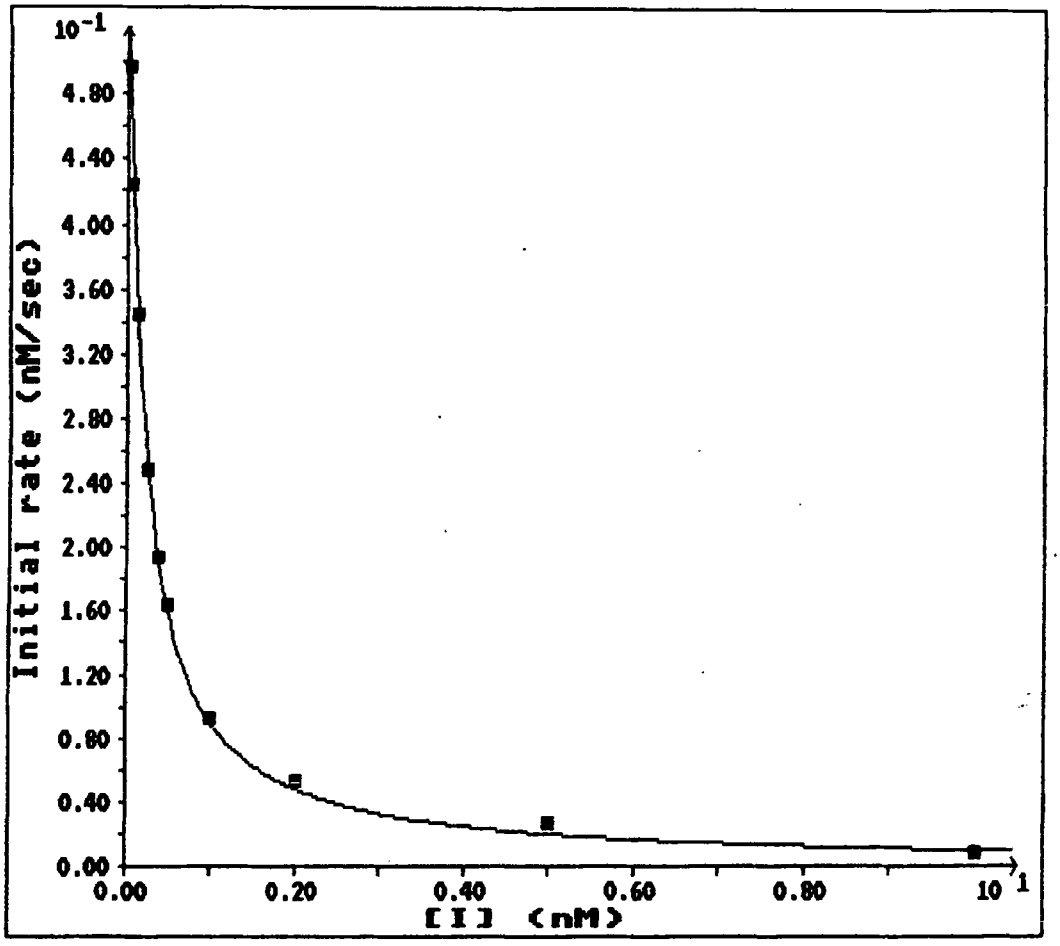
Figure 6.4 The Determination of Ki_{app} for CT435 against gelatinase-A

Gelatinase-A (0.06 nM) was incubated at 23°C in peptide assay buffer (2.49 ml) together with CT435 at concentrations ranging from 0 - 10 nM. After 4 hours, 10 µl of the substrate (QF24) was added to give a final concentration of 1.4 µM. Rates of hydrolysis were determined over the first two minutes. The apparent Ki (Ki_{app}) was determined using Enzfitter (Leatherbarrow, 1987) by a non-linear regression of the following equation;

$$v_s = \left(\frac{v_o}{2E_o}\right)\left[\sqrt{(Ki_{app} + I_t + E_t)^2 + 4E_t Ki_{app}} - (Ki_{app} + I_t + E_t)\right]$$

v_o and v_s are the steady state rates of the reaction in the absence and presence of inhibitor, respectively, E_t and I_t are the total concentrations of enzyme and inhibitor, respectively. Under conditions of $S << K_m$ the Ki_{app} value approximates to the Ki.

Figure 6.4 The Determination of Ki_{app} for CT435 Against Gelatinase-A



Ki_{app} FOR CT435 AGAINST GELATINASE-A (4 HRS PRE-INC)

High affinity inhibition
Simple weighting
[enzyme] = 6.00000E-02

Variable	Value	Standard Error
Apparent Ki [nM]	1.83730E-01	8.17623E-03
Rate-inhibitor	5.50133E-01	7.89140E-03

[E] ≥Ki. The rank order potency, however, is similar for the two substrates. Comparison of these values with the corresponding IC_{50}'s emphasises the importance of determining Ki's to establish SARs for highly potent compounds.

(c) Pre-steady-state kinetics

Progress curves of the response of gelatinase-A to CT989 confirm that inhibition is time-dependent (see Fig. 6.6) and occurs at concentrations which are comparable to those of the enzyme. Progress curves were analysed by two procedures according to the inhibitor concentration range tested.

Method 1

CT435 (Ki ~ 0.2 nM) was studied under conditions in which inhibitor concentration was at least 10 fold higher than that of gelatinase-A. CT435 concentrations were varied in the range of 0.5 nM - 10 nM in the presence of 0.05 nM enzyme. Under these conditions < 10% of the inhibitor can be bound to enzyme at any time and so free [inhibitor] approximates to total [inhibitor] and the pseudo-first order rate constant k_{obs} can be determined from equation (4)

$$P = v_s t + (v_o - v_s)(1 - \exp[-k_{obs} t])/k_{obs} \qquad (4)$$

where P is the concentration of product, v_o and v_s are the initial and final steady-state velocities, respectively for enzyme catalysis in the presence of inhibitor (Morrison and Walsh, 1988).

The second order rate constant for association, k_{on} (equivalent to k_1 in mechanism A), was determined from a plot of k_{obs} (y - axis) against [I] (x - axis) according to the equation,

$$k_{obs} = k_{on} I + k_{off} \qquad (5)$$

The unimolecular rate constant k_{off} (dissociation rate constant) (equivalent to k_2 in mechanism A) was determined from the vertical intercept. Ki_{app} can be determined from the intercept on the horizontal axis. As there can be a high margin of error associated with the calculation of k_{off} by this procedure (particularly for inhibitors exhibiting slow dissociation rates) this value was verified by using the relationships,

$$Ki = k_{off}/k_{on} \qquad (6) \quad \text{and} \quad k_{off} = k_{obs}\left(\frac{v_s}{v_o}\right) \qquad (7)$$

Results from the CT435 study are shown in Fig. 6.5 The linearity of the dependence of k_{obs} on CT435 concentration provides no evidence for a situation other than a simple bimolecular interaction (mechanism A). A Ki value of 0.188

nM was determined from the intercept on the horizontal axis and this result is consistent with that obtained from steady-state analysis (0.18 nM).

Method 2

Many of the inhibitors have substantially lower Ki values than CT435. For such compounds the rate of inhibition at concentrations greater than 10 fold higher than the enzyme concentration was too rapid to permit the use of method 1 for data analysis. In order to permit the use of lower inhibitor concentrations the equation must include terms which allow for the depletion of free inhibitor. Appropriate equations, have been described for mechanism A by Williams *et al.* (1979) in a study of the binding of folate analogues to dihydrofolate reductase.

$$v = \frac{v_s + [v_o(1-\gamma) - v_s]\exp(-\lambda t)}{1 - \gamma\exp(-\lambda t)} \qquad (8)$$

which on integration becomes,

$$P = \frac{v_s t + (1-\gamma)(v_o - v_s)}{\lambda t} \ln\left(\frac{1-\gamma\exp(-\lambda t)}{1-\gamma}\right) \qquad (9)$$

where the terms P, v_o and v_s are as described above and the parameters γ and λ are described by the equations,

$$\gamma = E_t\left(1 - {}^{v_s}\!/_{v_o}\right)^2 \Big/ I_t \qquad (10)$$

$$\lambda = \frac{k_{on}k_m}{(k_m + S)\sqrt{\left[\left(Ki_{app} + E_t + I_t\right)^2 - 4E_tI_t\right]}} \qquad (11)$$

in which E_t and I_t are total enzyme and inhibitor respectively and S is the concentration of substrate. The terms Ki_{app} and k_{on} have been described previously. Since S << K_m equation **(11)** reduces to,

$$\lambda = k_{on}\sqrt{\left[\left(Ki_{app} + E_t + I_t\right)^2 - 4E_tI_t\right]} \qquad (12)$$

This form of the equation was used for analysing data from inhibitors with Ki's of the same order of magnitude as the enzyme concentration such as CT1746. Where Ki << E_t and I_t (CT989) this reduces further to,

$$\lambda = k\sqrt{\left[\left(E_t + I_t\right)^2 - 4E_tI_t\right]} \qquad (13)$$

from which a value for k_{on} can be determined directly. A value for k_{off} for CT989 was determined by using either equation **(6)** together with a Ki_{app} determined under steady-state conditions or **(7)** at inhibitor concentrations suitable for the determination of the rate constant, k_{obs}.

Figure 6.5 Analysis of pre-steady-state kinetics of CT435 against gelatinase-A

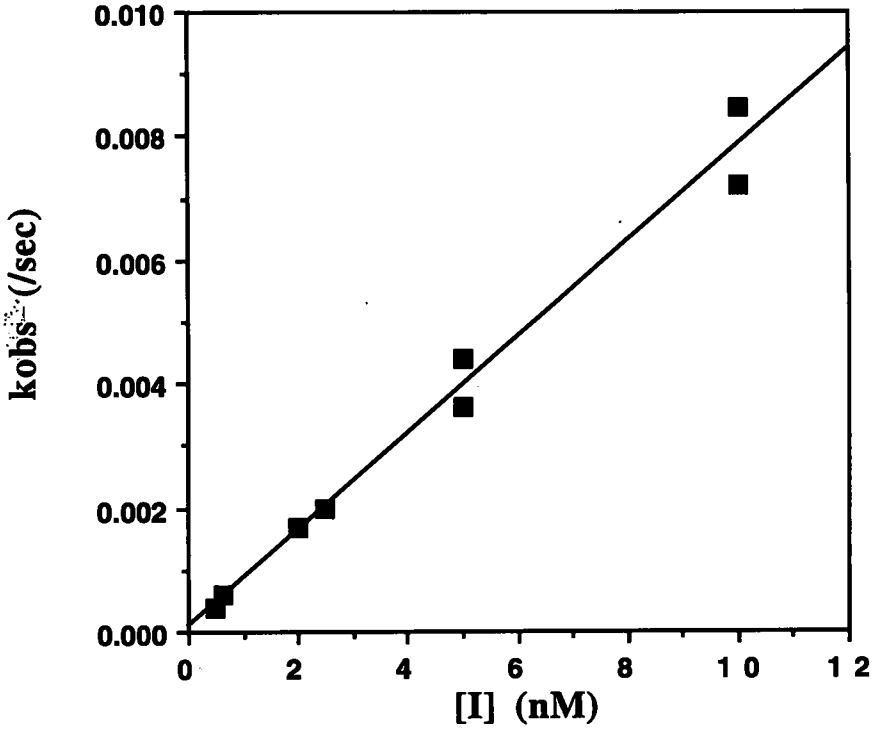
CT435, at concentrations ranging from 0.5 - 10 nM, was added to QF24 (1.4 μM) in peptide assay buffer (2.49 ml). The reaction was initiated by the addition of gelatinase-A (0.05 nM) and then hydrolysis of the substrate was monitored continuously until a steady-state velocity was attained. Progress curves were analysed using the Enzfitter programme and k_{obs} an apparent first order rate constant describing the establishment of equilibrium between enzyme-inhibitor complexes was determined from the integrated rate equation-

$$P = v_s t + (v_o - v_s)(1 - \exp[-k_{obs} t])/k_{obs} \qquad (4)$$

where P is the concentration of product, and v_o and v_s are the initial and final steady-state velocities, respectively for enzyme catalysis in the presence of inhibitor (Morrison and Walsh, 1988).

Figure 6.5

Analysis of pre-steady-state kinetics of CT435 against gelatinase-A



- Intercept on y-axis = 1.44e-04/sec (koff)
- Slope = 7.67e+05/M/sec (kon)
- Intercept on x-axis = 0.188 nM (Kiapp)

Table 6.2 shows the result of such an analysis for the inhibitor CT989 and the corresponding progress curves are presented in Fig. 6.6. The progress curves for CT989 were generated essentially as described in **method 1** using inhibitor concentrations ranging from 0.075 - 1 nM together with 0.032 nM gelatinase-A. A mean value for k_{on} was determined from the λ values generated by Enzfitter analysis of the progress curves using equation **(9)**. A mean value for k_{off} was determined from the progress curves at 3 inhibitor concentrations 0.3, 0.5 and 1 nM which satisfy the criteria [I] ≥ 10 times [E] that permits analysis by **method 1.**

The results of pre-steady-state analysis on a selection of inhibitors are presented in Table 6.1. The high affinity of inhibitors such as CT962, CT989 and CT1166 appears to be a function of their exceptionally slow rates of dissociation. Replacement of the hydroxamic acid zinc binding group (CT989) with a carboxylic acid (CT976) had little effect on the k_{on} but increased the k_{off} by 30 fold and this is reflected in the comparative Ki's of the two compounds. Comparison of the dissociation rates of CT543 (3.02×10^{-4} s^{-1}) and CT962 (3.56×10^{-5} s^{-1}) indicates scope for manipulating this property by modifying the P_3' position. CT962 differs from CT543 only by the presence of an additional carbon in the P_3' hydrocarbon chain. Extension of the chain at this position did not affect the rates of association. Association rates were generally in the range of $2 - 7 \times 10^6$ $M^{-1}s^{-1}$ (Table 6.1 and data not shown). These values are within the range of diffusion controlled association rates. Consequently, by the criteria of Bartlett and Marlowe (1987) these inhibitors cannot be described as slow-binding. Inhibitors which are outside this range include CT435 and CT1746 which exhibited slower binding and faster binding rates, respectively. In the case of the former compound this may be due to an unfavourable fit of the P_3' phenethyl group into the S_3' binding pocket, since there is no reason to presume that it should have any more difficulty than other hydroxamates in displacing water from the active-site. The improved association rate of CT1746 may simply be a function of its reduced size and the absence of an extended P_3'/P_4' interaction. The lack of this side-chain also produces a significantly faster dissociation rate than those inhibitors with bulky P_3', resulting in a higher Ki value.

In summary, the very low Ki of this series of inhibitors is a property of their slow rates of dissociation and fast rates of association but differences due to k_{off}

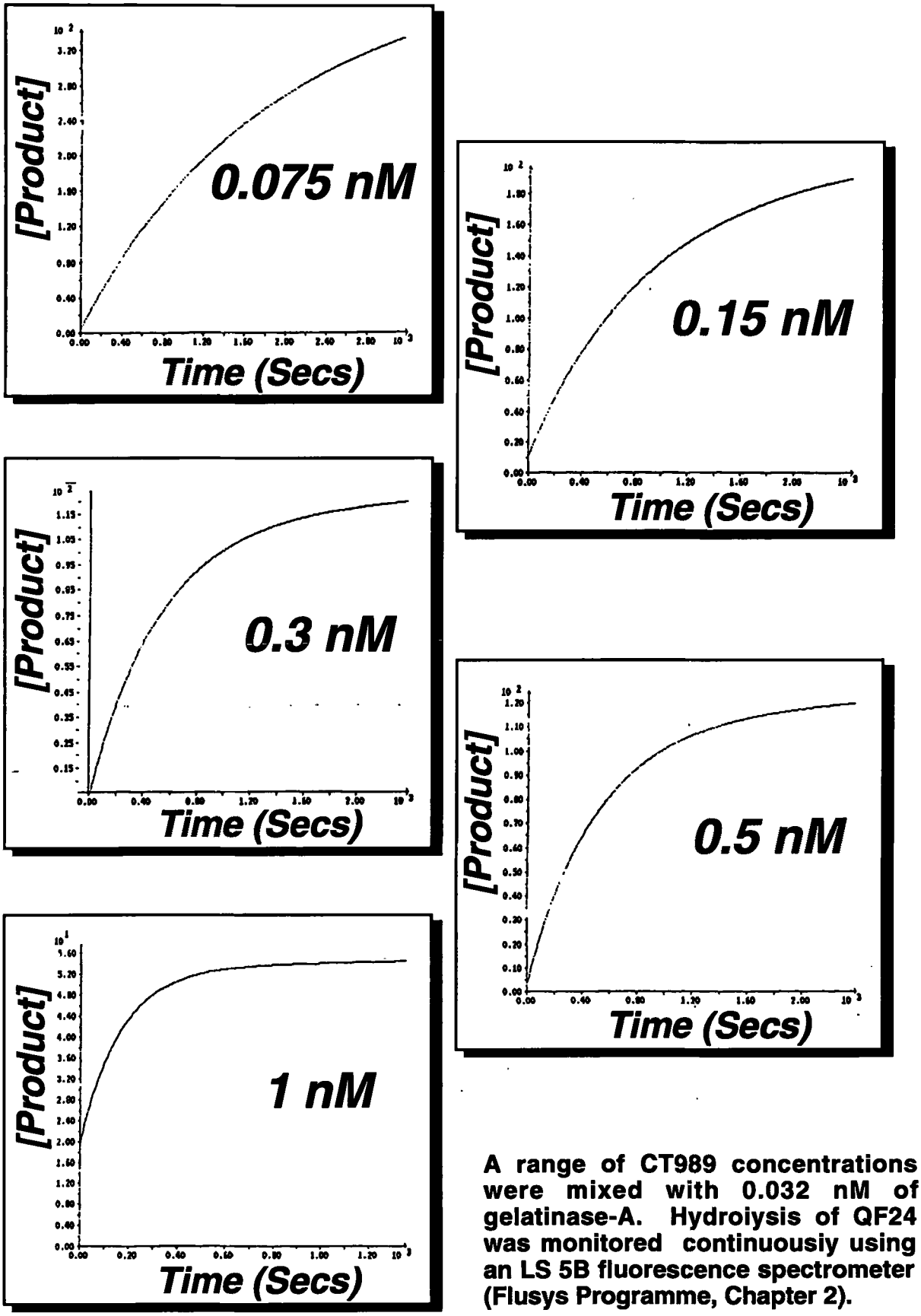
Table 6.1

A kinetic analysis of gelatinase-A inhibitors

Inhibitor	Gelatin (IC50) (nM)	Dnp-PLGLWAR (Ki) (nM)	QF24 (Ki) (nM)	QF24 k_{on} ($M^{-1}s^{-1}$)	QF24 k_{off} (s^{-1})	dissociation $t^{1/2}$ (mins)
CT435	2.5	0.30	0.18	7.67×10^{5} (a)	1.45×10^{-4} (a)	80
CT543	1.4	0.14	0.10	3.05×10^{6} (a)	3.02×10^{-4} (a)	38
CT962	< 1	0.04	0.01	3.56×10^{6} (b)	3.56×10^{-5} (b)	324
CT989	< 1	0.01	<0.01	7.10×10^{6} (b)	3.67×10^{-5} (b)	315
CT1166	< 1	0.01	<0.01	5.45×10^{6} (b)	4.22×10^{-5} (b)	273
CT1746	< 1	0.07	0.03	1.60×10^{7} (b)	6.40×10^{-4} (b)	18
CT976	13.1	0.9	0.45	2.32×10^{6} (a)	1.05×10^{-3} (a)	11

Values were determined as described in the text (section 6.2:1{ii}). Rate constants were determined using method 1 (a) or method 2 (b). The dissociation half-life ($t_{1/2}$) was calculated from the relationship ; $t_{1/2} = 0.693/k_{off}$

Figure 6.6 *Slow-binding Inhibition of Gelatinase-A by CT989*



A range of CT989 concentrations were mixed with 0.032 nM of gelatinase-A. Hydroiysis of QF24 was monitored continuousiy using an LS 5B fluorescence spectrometer (Flusys Programme, Chapter 2).

Table 6.2 The pre-steady-state kinetics of gelatinase-A inhibition by CT989

$$K_{on} = \lambda \Big/ \sqrt{\left[(E_t + I_t)^2 - 4E_tI_t\right]} \quad \text{and} \quad K_{off} = K_{obs} \cdot \frac{V_s}{V_o}$$

[CT989] nM	λ (10^3)	[a] ([E] +[I])^2	[b} 4[E]. [I]	[a] - [b]	k_{on} ($M^{-1}s^{-1}$)
0.075	0.43	0.0114	0.0096	0.0018	10.0×10^6
0.15	0.97	0.033	0.0192	0.0138	8.3×10^6
0.3	1.9	0.11	0.0384	0.0718	7.0×10^6
0.5	1.94	0.283	0.064	0.219	4.1×10^6
1.0	6.1	1.065	0.128	0.937	6.3×10^6

[CT989] nM	v_o	v_s	k_{obs} (s^{-1})	k_{off} (s^{-1})
0.3	0.219	0.0038	0.002	3.47×10^{-5}
0.5	0.194	0.003	0.003	4.60×10^{-5}
1.0	0.207	0.001	0.0061	2.95×10^{-5}

Mean $k_{on} = 7.13 \pm 2.2 \times 10^6 M^{-1}s^{-1}$ **Mean $k_{off} = 3.67 \pm 0.84 \times 10^{-5} s^{-1}$**

may be achieved by modifications at the P_3' position. Extending the hydrocarbon chain and increasing the size of the group at this position appears to increase either the strength or number of contacts made with the enzyme. Binding of the hydroxamic acid also appears to play a key role in preventing dissociation of the inhibitor-enzyme complex. These studies provided no evidence to suggest a mechanism of slow-binding involving significant intermediate states (mechanism B) although the experimental difficulty in establishing the relationship of v_i to v_o at high inhibitor concentrations means that such a mechanism cannot be discounted.

6.2:2 Requirements for inhibitor binding

(i) Binding of CT435 to gelatinase-A ($E^{375}\rightarrow D$)

Zinc metalloproteinases with mutations of the catalytic glutamic acid residue exhibit significantly reduced activity (Devault *et al.* 1988; Sanchez-Lopez *et al.* 1988). This has been demonstrated using gelatinase-A by Crabbe *et al.*, (1994a) and its importance in the catalytic process makes it a potential target for inhibitors. In order to study the role of the glutamic acid in inhibitor binding, the Ki_{app} for CT435 was determined against gelatinase-A($E^{375}\rightarrow D$), using QF24. This was possible because this form of gelatinase retains 10% of the catalytic activity (k_{cat}/K_m = 5.7 x 10^4 $M^{-1}s^{-1}$, Crabbe *et al.*, 1994a) of the parent. If E^{375} does have a significant interaction with CT435, the Ki_{app} would be expected to be higher than that for the parent.

Gelatinase-A($E^{375}\rightarrow D$) or gelatinase-A (0.2 nM) were incubated in peptide assay buffer (2.49 ml) for 2 hours at 23°C in the presence of CT435 at concentrations ranging from 0 - 4 nM. A similar study was conducted in parallel using gelatinase-A at a concentration of 0.02 nM (standard Ki assay conditions). Reactions were initiated by the addition of QF24 (1.4μM). Ki_{app} values were determined using the Enzfitter programme (tight-binding inhibition). CT435 had Ki_{app} values of 0.155 nM against gelatinase-A($E^{375}\rightarrow D$) and 0.218 nM and 0.195 nM against 0.2 nM and 0.02 nM gelatinase-A, respectively.

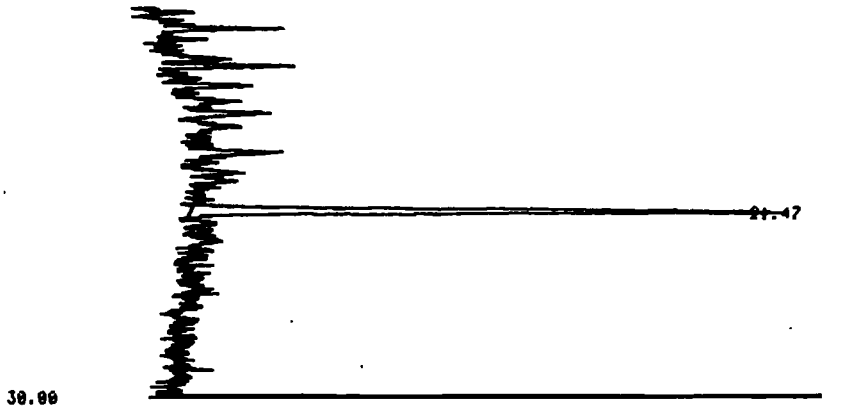
These results indicate that the catalytic glutamic acid residue E^{375} does not interact with the inhibitor.

Figure 6.7 CE analysis of CT543 binding to progelatinase-A and gelatinase-A

A solution of CT543 was added to either activated gelatinase-A, progelatinase-A (20μM) or assay buffer to give a final inhibitor concentration of 40 μM. Solutions were then incubated at 23°C for 2 hours. Free enzyme and complexed enzyme were subsequently removed by ultrafiltration (ultrafree 10 kDa molecular mass cut-off filter) and then residual inhibitor was bound to a sep-pak C_{18} reverse-phase cartridge and eluted as described in Chapter 2. Bound fractions were lyophilised prior to reconstitution in methanol. Samples were loaded electrokinetically for 30 secs at 30°C. Electrophoresis was carried out using a citrate buffer (pH 2.5) and peaks were detected at λ_{210}.

Figure 6.7 ***Analysis of CT543 Binding to Progelatinase-A and Geiatinase-A by CZE***

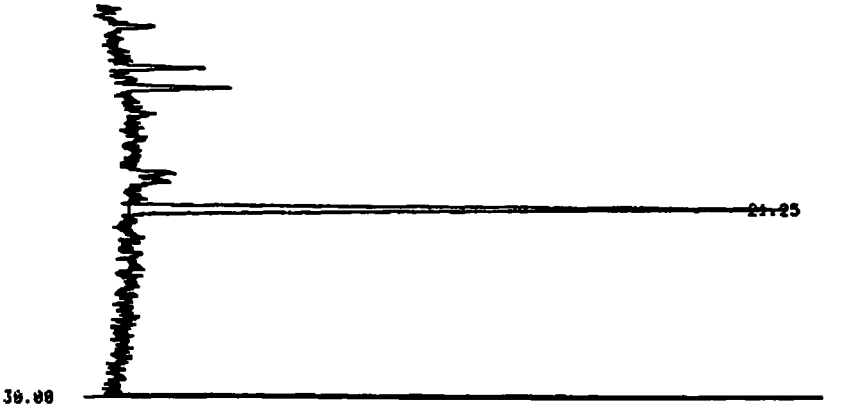
ACTIVE GELATINASE-A



Analysis 6.1Q
Sample A029 1217 051291 12:17 5/12/91
Method : A

Retn Time	Peak Ht	Peak Area	% Area	Peak No	Peak Start	Peak End
21.47	1.75	21.75	100.000	1	21.27B	21.87B

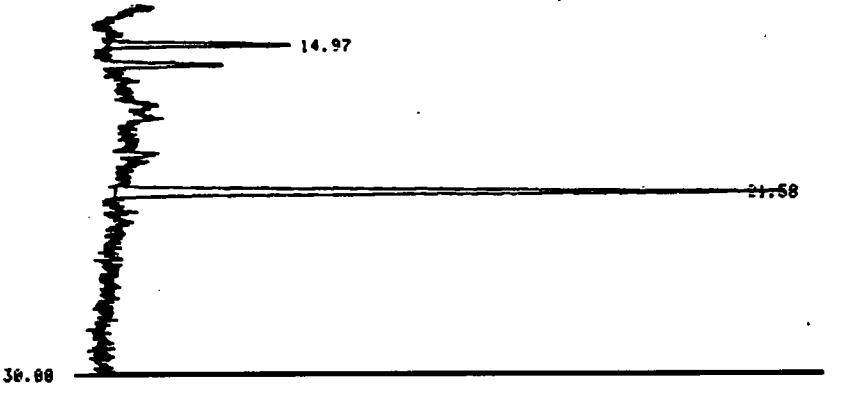
PROGELATINASE-A



Analysis 6.1Q
Sample A030 1400 051291 14:00 5/12/91
Method : A

Retn Time	Peak Ht	Peak Area	% Area	Peak No	Peak Start	Peak End
21.25	3.06	40.05	100.000	1	21.10B	21.68B

CONTROL



Analysis 6.1Q
Sample A028 1126 051291 11:26 5/12/91
Method : A

Retn Time	Peak Ht	Peak Area	% Area	Peak No	Peak Start	Peak End
09.85	0.61	2.18	4.225	1	9.72B	9.88B
14.97	0.84	9.14	17.724	2	14.80B	15.23B

(ii) CT543 binding to progelatinase-A

Low molecular mass inhibitors may be able to diffuse into the presumably masked active site of latent proenzyme. If this were to occur *in vivo* it might result in removal of inhibitor from the circulation before it can interact with active enzyme. The ability of a typical hydroxamic acid inhibitor, CT543 (see Table 5.5), to bind to progelatinase-A was investigated by ultrafiltration.

Progelatinase-A (20 μM) was activated using APMA (1 mM) for 7 hours at 23°C. A solution of CT543 was then added to either activated gelatinase-A, progelatinase-A or assay buffer. Following incubation free inhibitor was separated from bound using a combination of ultrafiltration and reverse-phase chromatography. Residual inhibitor was detected using capillary electrophoresis (CE). CT543 incubated with gelatinase-A, progelatinase-A or buffer produced relative peak areas of 21.75, 40.05 and 40.25, respectively (Fig. 6.7). Activated gelatinase-A, therefore, removed about 50% of the total inhibitor present which, assuming an initial enzyme:inhibitor ratio of 1:2 indicates stoichiometric binding. There was no difference in the peak areas of CT543 in the presence or absence of progelatinase-A which shows that the inhibitor does not bind to the proenzyme form of gelatinase-A. This result indicates that CT543 requires a freely accessible active-site for binding. This is corroborated by further studies in which addition of a 10 fold excess of progelatinase-A to a mixture of CT435 and gelatinase-A did not modify the ability of the inhibitor to titrate the active enzyme (data not shown).

6.3: Discussion

The interaction of Celltech MMP inhibitors with gelatinase was examined by kinetic analyses. The mechanism of the inhibitor-enzyme interaction was studied using both steady-state and pre-steady-state conditions. Ki's were determined using different substrates and subsequent analyses, using conditions designed to follow time-dependent inhibition, permitted the Ki to be broken down to its component terms, k_{on} and k_{off}. Finally, features of gelatinase-A required for inhibitor binding were established by testing the proenzyme and mutated enzyme which lacked the catalytic glutamic acid.

Throughout this work the assumption that inhibitors were both reversible and competitive inhibitors of MMPs has dictated the treatment of kinetic data. If this were not the case Ki's used for the construction of SARs would be invalid. Results in this chapter have, however, established that the inhibitors do have finite dissociation rates and the elevation of Ki_{app} in the presence of increasing concentrations of substrate effectively demonstrates the competitive nature of the interaction. The validity of these compounds as therapeutic inhibitors of gelatinases was tested using a physiological substrate, gelatin. Such assays were limited, however, by the high affinity of the inhibitors for gelatinase-A. The enzyme was titrated by inhibitor even at sub-nanomolar concentrations which is indicative of tight-binding. Additionally, the response of the enzyme to the presence of inhibitor occurred over many minutes which means that enzyme and inhibitor must be pre-incubated prior to assay. Steady-state analysis of the binding of CT435 to gelatinase-A demonstrated that under assay conditions equilibrium was established within four hours. The association kinetics of other inhibitors revealed that CT435 had a comparatively slow k_{on} value, thereby justifying the suitability of a four hour preincubation period for determining Ki's.

Tight-binding with time-dependent inhibition has also been reported in the neprilysin and ACE literature. The hydroxamate, idapril for example, has a Ki of 0.47 nM, k_{on} of 3×10^6 $M^{-1}s^{-1}$ and k_{off} of 1.4×10^{-3} s^{-1}. The typical range for the second order rate constant (k_{on}) of zinc metalloproteinase inhibitors is 10^5 - 10^7 $M^{-1}s^{-1}$ which is comparable to those presented in this chapter. Several studies were conducted to confirm the 'slow', tight-binding kinetics of these inhibitors described above. In one example (CT989), the time-course of the enzymic reaction was examined with or without preincubation of gelatinase-A and

inhibitor. Initiation of the reaction by the addition of enzyme resulted in a high initial velocity which gradually decreased to a lower steady-state velocity thereby producing a downward concave curve (Fig. 6.6). In another example, a high concentration of enzyme and inhibitor (CT1746) was incubated for four hours prior to initiation of the enzymic reaction by the addition of substrate. This produced an upward concave curve with a final steady-state velocity equivalent to that achieved under steady-state conditions (Fig. 6.1). This clearly indicates that equilibrium between gelatinase-A and inhibitor is not rapidly established under these assay conditions. It may be argued that time-dependent inhibition is not necessarily a consequence of slow-binding kinetics although the two have now become synonymous. These inhibitors clearly fulfil the criteria for slow-binding suggested by Schloss *et al.* (1988) i.e. potency increases on a time-scale that is readily followed by conventional assays. Indeed these authors include inhibitors with k_{on} values of the order of 10^6 $M^{-1}s^{-1}$ as slow-binding inhibitors. This classification leads to the inclusion of inhibitors with slow off rates (and which therefore take a long time to reach a steady-state velocity) and those that exhibit time-dependent inhibition as a consequence of the reaction conditions. Recognising the complexity of the process, Bartlett and Marlowe (1987) suggested a lower limit of 10^6 $M^{-1}s^{-1}$ for diffusion limited reactions and, indeed, there are many examples of inhibitors with k_{on}'s considerably lower than this. On these criteria many of the MMP inhibitors described in this study cannot be described as slow-binding, although CT435 does possess a k_{on} which is lower than this limit. The mechanism by which CT435 interacts with gelatinase-A was examined assuming a true slow-binding interaction. Whilst it is generally accepted that slow binding kinetics are a consequence of the fast formation of a collision complex followed by a slow isomerisation to a 'so-called' tightened complex (mechanism B) this proved to be difficult to prove experimentally. It was necessary to use inhibitor concentrations substantially lower than the Ki for the dissociation of the collision complex (where Ki* is the overall inhibition constant) and under these conditions this complex becomes kinetically insignificant. Accordingly, the linear dependence of the pseudo-first order rate constant k_{obs} on inhibitor concentration (Fig. 6.5) indicated that a single slow step leading to complex formation provided a satisfactory description of the kinetics.

Dissociation rate constants (k_{off}) were also established on the basis of a single enzyme-inhibitor complex. Examples were presented in which (1) k_{off}

could be determined from the y-axis intercept of a plot of k_{obs} against [I] (CT435) and (2) k_{off} was calculated from its relationship to k_{obs} and the initial and final steady-state velocities at several inhibitor concentrations (CT989). The former method could not be applied to CT989 because of its very slow rate of dissociation (dissociation half-life = 315 minutes). As the rate decreases the y-axis intercept approaches the origin at which point the reaction becomes essentially irreversible.

In this study, low Ki's were mainly a consequence of slow dissociation rates. CT1746 was an exception to this rule in that an increased association rate also contributed to its low Ki. This may be attributed to the absence of a large P_3' side-chain and its comparatively low molecular mass compared to inhibitors such as CT989 and CT1166.

Other studies were performed in order to discover which features of gelatinase-A might be important for inhibitor binding. Crystallographic studies have shown that inhibitors possessing a P_1' nitrogen form a hydrogen bond *via* this atom to the catalytic glutamic acid (Lovejoy *et al.*, 1994b). It may be argued that the nitrogen atom of hydroxamic acid may fulfil a similar role. This possibility was explored by studying the binding of a hydroxamic acid inhibitor to gelatinase-A, lacking the glutamic acid. Since the absence of the glutamic acid did not decrease the affinity of the inhibitor for gelatinase we can conclude that the hydroxamic acid does not interact with this residue. This has now been confirmed by co-crystallisation studies (Borkakoti *et al.*, 1994; Stams *et al.*, 1994).

Evidence for the requirement of a freely accessible active-site for inhibitor binding has been obtained by two independent methods. In one study, ultrafiltration was used to show that the addition of a concentrated solution of progelatinase-A to CT543 had negligible effect on the concentration of free inhibitor. Other studies examined the effect of progelatinase-A on the ability of inhibitor to titrate active enzyme as well as the effect of its presence on apparent Ki's (data not shown). Data from these studies are consistent with the ultrafiltration results and suggest that low molecular mass inhibitors cannot diffuse into the active-site.

In summary, the inhibitors interact with gelatinases in a reversible and competitive fashion. Additionally, they fulfil the criteria of Morrison *et al.*, (1982) for being classified as tight-binding. By using concentrations of enzyme and inhibitor of the same order of magnitude as the Ki's, the inhibitors may be

analysed using slow-binding kinetics. The high affinity of the hydroxamate inhibitors results from their slow rate of dissociation (long residency time) and a high rate of association with the enzyme. Finally, the catalytic glutamic acid at the active site of gelatinase-A is not involved in inhibitor binding but a freely accessible active site is required.

Chapter 7

CHAPTER 7

IN VIVO PROPERTIES OF SYNTHETIC MMP INHIBITORS

7.1: Introduction

Chapter 5 showed how classical structure-activity relationships (SARs) can be used to discover potent gelatinase inhibitors with a broad spectrum of properties against other MMPs. The structure of a compound also governs its behaviour in the body. Without information to the contrary we may assume that gelatinase inhibitors must provide continuous cover. For such drugs, an orally active formulation is the preferred route of administration. The level and frequency of dosing is determined largely by pharmacokinetic properties such as the systemic half-life. Since the relationship between the structure of a drug and its pharmacokinetic properties is different from that which determines inhibitory activity, the clinical candidate will probably result from a structural trade-off between the two. Studies described in this chapter, therefore, expand the inhibitor screening cascade to include pharmacological SARs.

Peptides or peptide-like substances exhibit notoriously poor pharmacokinetics (reviewed in Humphrey and Ringrose, 1986). Consequently, despite the fact that potent hydroxamic acid MMP inhibitors were discovered ten years ago there are few published reports describing their biological stability. Investigators at Sterling Winthrop (Singh *et al.*, 1993) reported SARs for a series of isobutyl (P_1'), tryptophan (P_2') inhibitors with a range of modifications at the P_3' position. Systemic half-lives in the rat were in the range of 6.9 minutes ($(CH_2)_4$ - COOH) to 43.7 minutes ($(CH_2)_2$ - S-$(CH_2)_2$ - N -$(CH_3)_2$) and percentage oral bioavailability was reported to range from 0 - 10 %. Biliary excretion was the major route of elimination either of the hydroxamate parent compound or of the corresponding carboxylic acid metabolite. Metabolic studies on BB94 (see Fig. 5.1) gave similar results (Yachetti *et al.*, 1993) but no pharmacokinetic details were presented. More recently Roche UK demonstrated that incorporation of a t-butylglycine at the P_2' position (eg. Ro31-9790) can lead to improved oral bioavailability in the isobutyl (P_1') class of inhibitors (Lewis *et al.*, 1993).

Research in the renin area has been particularly influential in the design of MMP inhibitors. A common theme arising from this work is the need for the correct balance of lipophilicity and hydrophilicity for oral bioavailability. To this

end, morpholines and other slightly basic groups are frequently used to provide a degree of water solubility to compounds.

Attention has been drawn to the rapid metabolism of hydroxamic acids in the 5-lipoxygenase area. Inhibitors of this enzyme are being developed as anti-inflammatory agents. Underivatised hydroxamates attached by methylene spacers to non-peptide structures were observed to convert rapidly to the corresponding carboxylic acid in rats (Summers *et al.*, 1987; Tateson *et al.*, 1988).

The ability of rats to metabolise hydroxamates is presumably the result of blood esterase action. This means that whole blood or plasma may be used, therefore, as an early screen for susceptible inhibitors. Moreover, it dissociates the effects of blood metabolism from that attributed to other organs. Consequently, in this study the metabolic activity of the blood from several animal species was tested using a range of extraction and detection techniques to measure inhibitors with diverse physiochemical properties. By comparing the activity of human blood to that of other species we can predict the extent of systemic metabolism at a stage well before the drug can be tested in humans.

Lark *et al.* (1990) described a rat pleural cavity assay which was used to allow synthetic inhibitors of gelatinase-A to be tested (section 2.4:3). It provides a primary *in vivo* screen to identify compounds with improved pharmacokinetic properties. Potential lead molecules can then be studied in more detail by measuring their blood levels in mice after intravenous administration. Since the resulting plots of Log_{10} concentration against time are essentially bi-exponential, pharmacokinetic treatment of the data employs a two-compartmental model (section 2.4:4) throughout this study.

The pharmacokinetic profile of an animal species may not be predictive of what may occur in other species, including humans. The ketomethylene tripeptide inhibitors described by Almquist *et al.* (1985), for example, exhibit half-lives of 10 minutes in the rat and 9 minutes in the rabbit, which shows that systemic half-life does not necessarily increase with the size of the species. A similar situation has been observed with non-peptide hydroxamic acids. The 5-lipoxygenase inhibitor zileuton, for instance, exhibits half-lives of 2.4 hrs, 4 hrs, 7.5 hrs and 0.3 hrs in rat, sheep, dog and monkey, respectively. Consequently, the following pharmacokinetic studies have been performed in mice, rats and rabbits.

Few orally active MPIs have been described and none with the P_1' aryl group which confers gelatinase potency and selectivity. It is nonetheless an important requirement for MMP inhibitors. It is commonly presented as the term "percentage oral bioavailability" which relates the amount of drug entering the systemic circulation *via* the oral route to that present after intravenous administration. Methods for calculating this value are presented in section 2.4:4. The pleural cavity identified a number of orally active compounds for pharmacokinetic analysis in mice and rats. Ro31-9790 was used as a control for these studies since its bioavailability in the rat has been reported.

The bioavailability studies described above were performed using non-radiolabelled samples. Concentration-time plots were, therefore, generated by titrating extracted activity against gelatinase-A. This technique is very sensitive but may not discriminate between parent drug and active metabolites. The results of these studies will, therefore, be discussed in the light of recent data obtained from [^{14}C]-labelled CT1746.

7.2 : Results

7.2:1 The blood stability of MMP inhibitors

Studies of hydroxamic acid inhibitors of 5-lipoxygenase (Tateson *et al.*, 1988) have suggested that rodents may possess plasma esterases capable of converting the hydroxamic acid to the corresponding carboxylic acid. The comparative lack of inhibitory activity of the latter was shown to produce a rapid loss in biological activity which is reflected in the poor systemic half-lives of these compounds. Peptide hydroxamates may also act as substrates for these enzymes and their susceptibility may be tested *ex vivo* using whole blood or plasma. A range of methods have been developed to measure the presence of parent drug in biological fluids, examples of which will be presented in the following *ex vivo* studies.

(i) The stability of CT543 and CT550 in mouse blood

CT550 and CT543 were incubated at 37°C in mouse blood containing an anti-coagulant. Samples were removed after 0, 2 and 5 hours for the preparation of plasma fractions. Inhibitors were isolated using solid-phase extraction prior to analysis by CE. Table 7.1 shows that after 5 hours, 44% and 46% of CT543 and CT550 remained, respectively. The decrease in levels of parent compound was not accompanied by a corresponding increase in any other peak. Since electrokinetic loading discriminates against negatively charged species it is possible that carboxylic acid metabolites, for example, would not be detected. This study suggests CT550 and CT543 are unstable in mouse blood although the rate of removal of parent compound is comparatively slow.

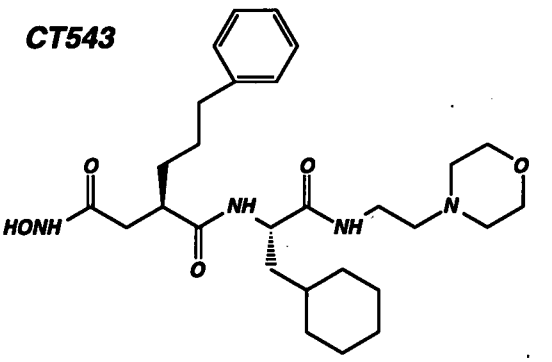
(ii) The stability of CT420 in human plasma

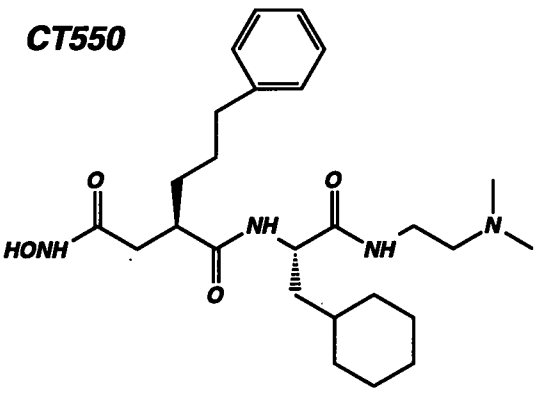
The stability of the P_1' isobutyl inhibitor CT420 was tested using human plasma. Parent compound was detected using reverse phase HPLC after ethyl acetate extraction. Results were compared to those for a corresponding sample incubated in water (Table 7.2).

Table 7.2 shows that CT420 is stable at 37°C in human blood for at least 4 hours. Additional HPLC data indicates that the parent compound remains in the blood after 24 hours incubation (data not shown). This study indicates that

Table 7.1 The stability of CT543 and CT550 in mouse blood

Sample	Time (hrs)	Peak area	% remaining
CT543	0	2636	100
	2	1729	66
	5	1171	44
CT550	0	3601	100
	2	1849	51
	5	1677	46



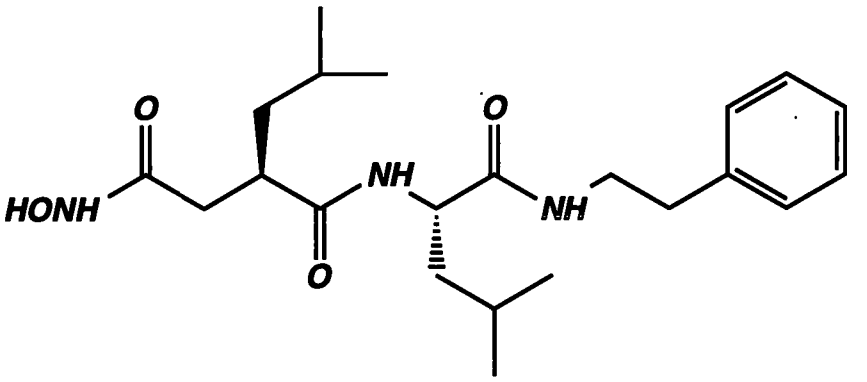


CT543 and CT550 (100 µg/ml) were added to 3.3 ml of citrated whole mouse blood. At 0, 2 and 5 hours plasma was prepared and passed over a C_{18} sep-pak reverse-phase cartridge. Bound inhibitor was eluted with 70% acetonitrile/0.1% TFA and then dried by vacuum centrifugation. Reconstituted (0.5 ml methanol) material was filtered prior to electrokinetic application (5Kv, 30 secs) to an ABI 270A CE. Samples were electrophoresed in citrate buffer, pH 2.5 at 39°C. Peaks were detected at λ_{210} nm.

Table 7.2 The stability of CT420 in human plasma

Time (hrs)	water (area)	plasma (area)	plasma/water (%)
0	536188	652901	122
0.25	591251	712568	121
4	587629	701157	119

CT420



CT420 (250 μg/ml) was added to 1 ml of human plasma. At 0, 15 mins and 4 hrs inhibitor was extracted into ethyl acetate and dried by vacuum centrifugation. Reconstituted sample (0.5 mls of 5% methanol/0.1% TFA) was applied to a Dynamex C_{18} reverse-phase column. CT420 was separated from contaminating species using a 10 - 40% acetonitrile gradient with detection at λ_{257} nm. Peak areas represent the mean of 3 determinations.

Table 7.3 The stability of CT1746 in mouse and rabbit blood

Species	Extraction	Concentration (μM)		%remaining
		0 hours	1 hour	at 1 hour
Mouse	**Ethylacetate**	**1.09 ± 0.09**	**0.88 ± 0.02**	**81**
Mouse	**Methanol**	**1.21 ± 0.1**	**0.934 ± 0.07**	**77**
Rabbit	**Ethylacetate**	**0.86 ± 0.12**	**0.845 ± 0.02**	**99**
Rabbit	**Methanol**	**1.13 ± 0.03**	**0.742 ± 0.04**	**66**

CT1746 (1μM) was added to duplicate samples of whole mouse or rabbit blood (heparinised) previously warmed to 37°C. At 0 hrs and 1 hr samples (0.5ml) were removed either directly into ethyl acetate (2ml) or centrifuged to produce the plasma fraction. Plasma samples (0.25ml) were precipitated on ice with 4 volumes of methanol. Ethylacetate samples (1ml) were lyophilised by speedvac and reconstituted in assay buffer + 5% methanol. Plasma proteins were precipitated from the methanol samples by centrifugation and then 1ml of the methanol was removed, lyophilised by speed vac and then reconstituted in assay buffer/methanol as above. Samples were diluted appropriately for titration against gelatinase-A using the QF24 assay. Fluorescence recordings were read off an appropriate standard titration curve.

human blood does not metabolise hydroxamate inhibitors as efficiently as rodent blood.

(iii) The stability of CT1746 in mouse and rabbit biood

The hydroxamate inhibitor CT1746 has shown some interesting *in vivo* characteristics which will be described in section 7.2:3 but there is evidence of instability in rodent blood (Dr. G. Curtiss, personal communication). Stability was examined in the blood of two species, mouse and rabbit, after ethyl acetate extraction or methanol precipitation. The former should discriminate against hydrophilic metabolites. This study was performed using an inhibitor concentration of 1μM which is comparable with concentrations observed *in vivo.* Inhibitor levels were estimated by titration against gelatinase-A for maximal sensitivity.

CT1746 was added to heparinised blood which had been pre-warmed to 37°C. Samples were removed either immediately or after 1 hour and then inhibitor was extracted and quantified as described above.

Table 7.3 shows that the recovery of activity was similar for both methods of extraction. This result suggests that the compound is not extensively modified. Approximately 80% of CT1746 related activity was recovered after 1 hour in the blood of both species.

CT1746 exhibits some instability in both mouse and rabbit blood which shows that the ability to modify these inhibitors is not confined to rat blood.

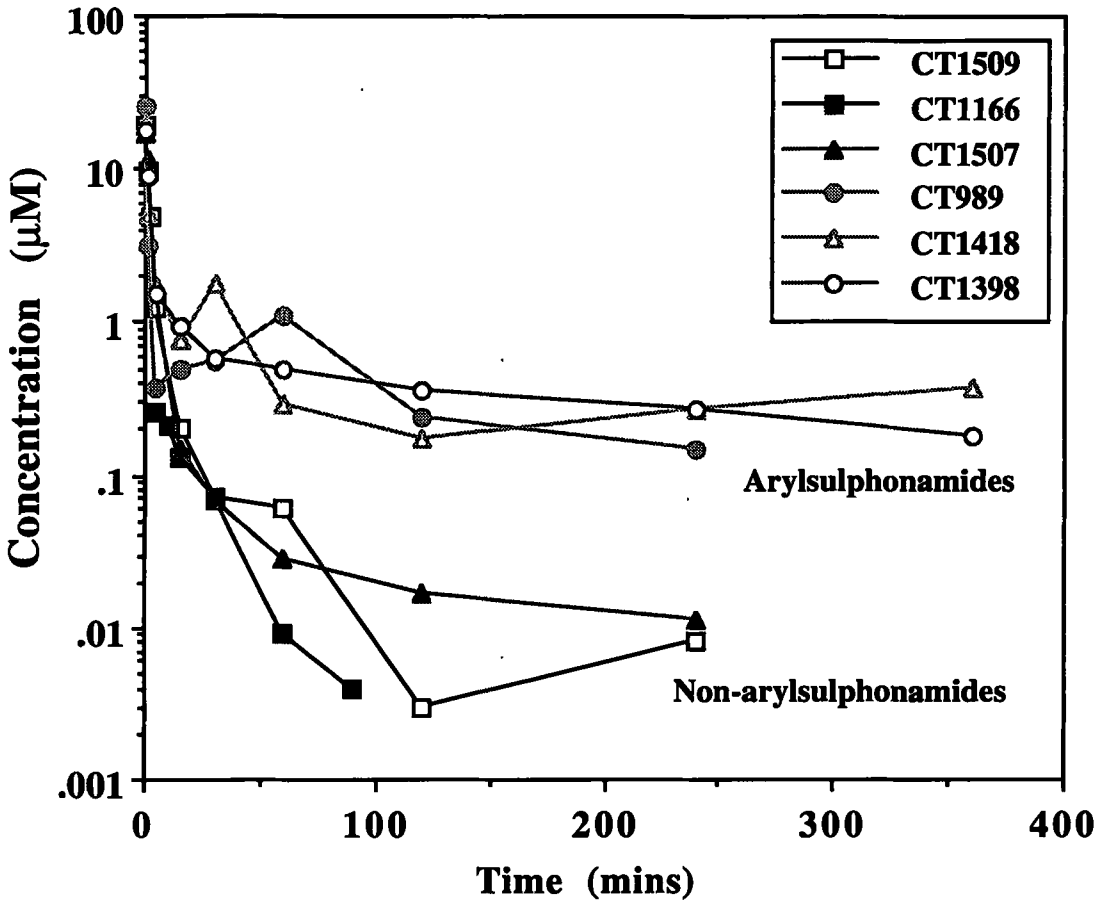
7.2:2 The pharmacokinetics of MMP inhibitors

(i) The pharmacokinetic properties of aryisuiphonamide inhibitors in mice

A long systemic half-life may be particularly important if a drug is to maintain 24 hour cover in humans. This parameter can be calculated by measuring the levels of inhibitor in the blood at various times after intravenous (i.v.) administration of a bolus dose. In the following study systemic concentrations of the CT989 series of arylsulphonamides (CT989, CT1398 and CT1418) were compared to those obtained from three non-arylsulphonamide inhibitors (CT1166, CT1507 and CT1509).

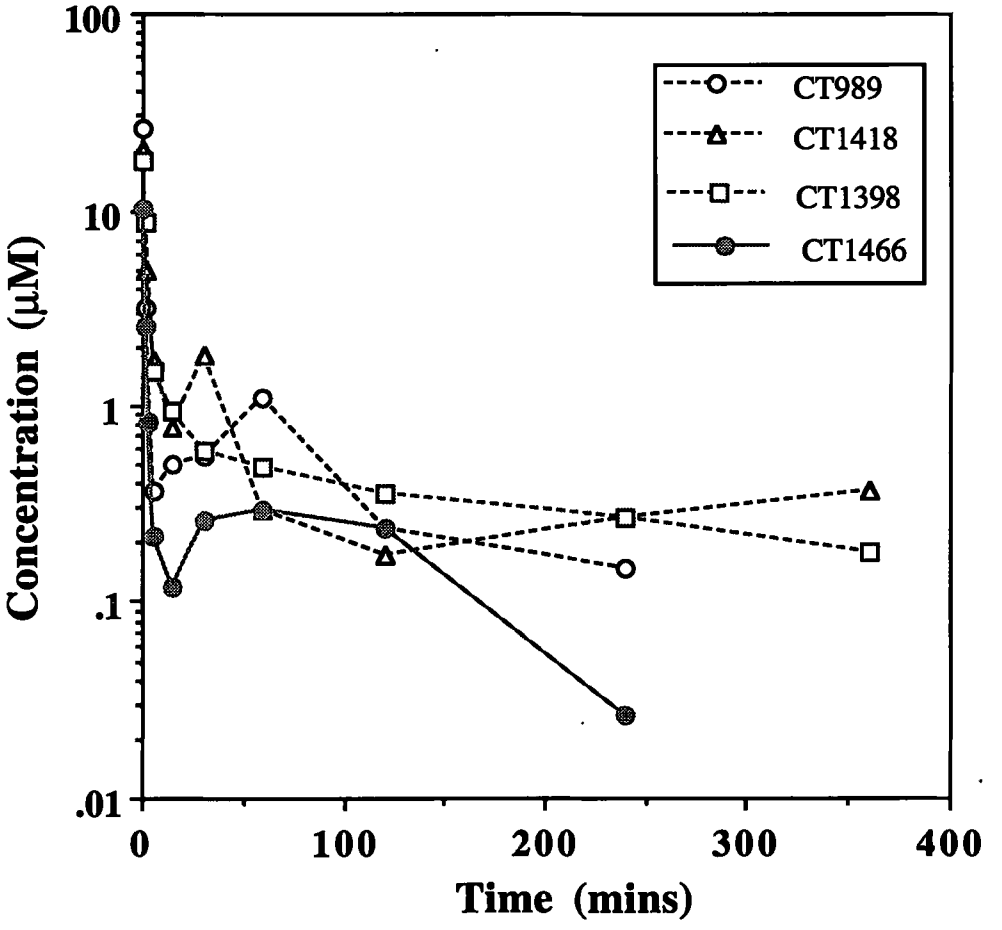
Compounds were administered intravenously at a dose of 4 mg/kg to groups of 5 animals (25g). At various times the animals were sacrificed and the

Figure 7.1 Inhibitor levels in mouse blood following intravenous administration



Compounds were administered intravenously at a dose of 4 mg/kg to groups of 5 animals. At times ranging from 10 secs to 6 hrs the animals were sacrificed and inhibitor was extracted from 0.5 ml of blood using ethyl acetate (2 ml). The concentration of inhibitor-related activity was determined by titration against gelatinase-A. Log_{10} concentration was then plotted as a function of time for each inhibitor. The plots represent the mean of 5 indivdual determinations.

Figure 7.2 The role of hydroxamic acid in the clearance of arylsulphonamides in mice



CT1466 was administered intravenously at a dose of 4 mg/kg to groups of 5 animals. At times ranging from 10 secs to 4 hrs the animals were sacrificed and inhibitor was extracted from 0.5 ml of blood using ethyl acetate (2 ml). The concentration of inhibitor-related activity was determined by CE analysis using vacuum application. Samples were electrophoresed in a buffer of 20 mM Tris / 28.8mM borate / 0.68 mM EDTA / 30% methanol, pH 9.0. The resulting plot was then overlaid on the blood profiles for CT989, CT1398 and CT1418 (Fig. 7.1).

concentrations of inhibitory activity were determined in ethyl acetate extracts of whole blood.

Fig. 7.1 shows that all the inhibitors possess a vary rapid α-phase distribution with half-lives ($t_{1/2}$) typically shorter than 1 minute. Analysis of the raw data reveals this to be particularly evident for the arylsulphonamide, CT989 which has an $t_{1/2}$ (α) of only 0.39 minutes. Only 1.4% of the CT989 activity present 10 seconds post-administration could be detected after 5 minutes. The corresponding percentages for CT1509, CT1507, CT1418 and CT1398 are in the range of 6.4% - 9.4%. It is apparent from the blood profiles that much higher levels of the arylsulphonamides are retained in the blood from 15 minutes onwards. After 1 hour, for example, only 0.4% of the initial concentration (measured at 10 secs) of CT1509 and CT1507 is still detectable, whereas, the corresponding figures for CT989 and CT1398 are 3% and 4%, respectively. An additional distinguishing feature of CT989 and CT1418 is the presence of a significant increase in inhibitor concentration between 30 minutes and 1 hour.

The β–phase half-lives were estimated to be in the range of 15 - 20 minutes for the non-arylsulphonamides. The concentration-time plots show that the $t_{1/2}(\beta)$ is significantly higher for the aryisulphonamide series. A half-life of 120 minutes was calculated for CT989 but this figure is likely to be inaccurate since the kinetics of its elimination appears to be far more complex than can be described by a simple two-compartmental model.

The prolonged $t_{1/2}(\beta)$ of the arylsulphonamides is not consistent with pleural cavity assay data (data not shown). This assay could not distinguish between CT989 and CT1166 although concentrations of the former appear to be considerably higher in the blood. This indicates that the pleural cavity is inaccessible to a fraction of the ethyl acetate extracted CT989.

The $t_{1/2}(\beta)$ values are comparable to those described by Singh *et al.* (1993) for a structurally related series of collagenase inhibitors tested in rats (range, 6.9 - 43.7 minutes). This study provides further evidence that peptide-hydroxamate inhibitors of MMPs exhibit relatively poor pharmacokinetics in rodents.

(ii) The role of hydroxamic acid in the blood clearance of arylsulphonamides in mice

The hydroxamic acid provides a major contribution to the inhibitory activity of MMP inhibitors. For this reason its presence is ubiquitous in the design of most active compounds. The hydroxamate may also play a major role in promoting the rapid clearance of these compounds since an extensive programme of side-chain modifications has not produced a compound with markedly altered pharmacokinetics.

Its role in the pharmacokinetics of the arylsulphonamides was examined by removing the entire zinc-binding ligand to produce CT1466. If the hydroxamic acid dictates the elimination rate of the arylsulphonamides, one would expect considerably higher concentrations of CT1466 to be retained in the blood at later times.

CT1466 was administered at 4 mg/kg (i.v.) to groups of 5 mice. At various times the animals were sacrificed and inhibitor was extracted from the blood. The concentration of CT1466 was determined in individual samples using CE analysis. The data were plotted as described above and the resulting plot was overlaid on the blood profiles of CT989, CT1398 and CT1418 (Fig. 7.2).

CT1466 displayed a rapid α distribution phase which was reflected in only 2.1% of the initial inhibitor concentration being detectable in the blood after 5 minutes. Like the other arylsulphonamides blood concentrations appeared to increase after 30 minutes. At no point were the blood concentrations of CT1466 significantly higher than those of other members of this series. The overall shape of the concentration curve was similar to that produced by other arylsulphonamide inhibitors. These results indicate that the arylsulphonamide rather than the hydroxamic acid is the major determinant of the pharmacokinetics of this series of inhibitors.

(iii) The properties of hydroxamic inhibitors in the rabbit

Pharmacokinetic data obtained from mice is of limited accuracy because the low blood volume makes sequential blood sampling from a single animal, impractical. Additionally, the pharmacokinetics of this class of molecule has only been described in mouse and rat (see above) which are both rodents. Consequently, the pharmacokinetics of a broad spectrum hydroxamate (CT435) and a selective gelatinase inhibitor (CT989) was studied in the rabbit.

Figure 7.3 Concentration-time plots of CT435 and CT989 in rabbit blood following i.v. administration

CT435 (2mg/kg) and CT989 (10mg/kg) were administered *via* the femoral vein to two rabbits. Blood samples (1ml) were then removed directly into ethyl acetate at times ranging from 0 - 6 hrs. The concentration of extracted inhibitor was then determined by activity assay (3 determinations) against gelatinase-A and the results verified by CE (CT989 only).

Figure 7.3 Concentration-time plots of CT435 and CT989 in rabbit blood

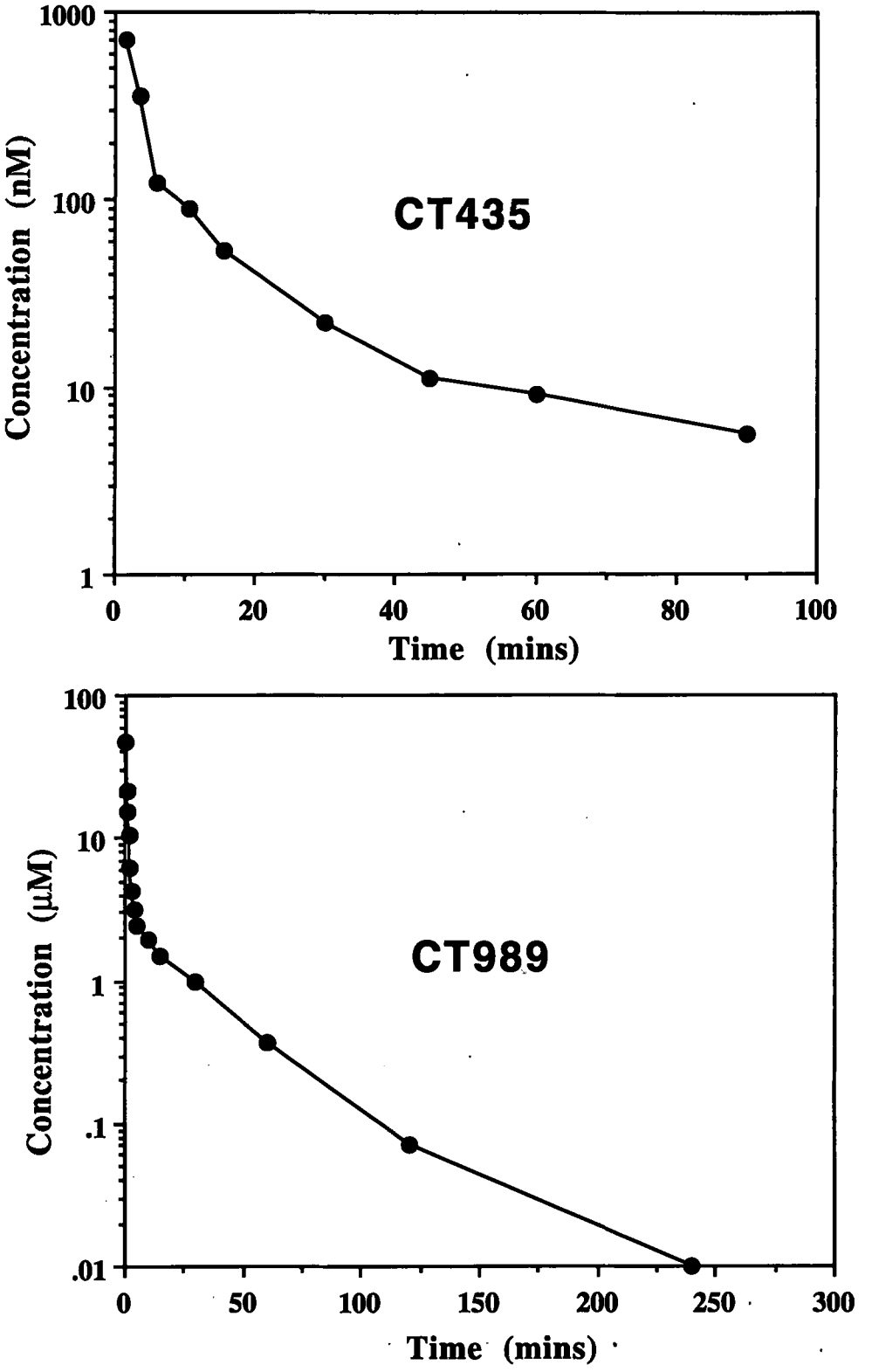


Table 7.4

Levels of CT989 in rabbit blood following i.v. administration of a dose of 10mg/kg

TIME (MINS)	CONCENTRATION	
	[μ M]	[μ g/ml]
0.45	48.0	29.0
0.75	21.6	13.0
1.08	15.0	9.0
1.5	10.6	6.3
2	6.1	3.7
3	4.3	2.6
4	3.2	1.9
5	2.4	1.4
10	1.9	1.1
15	1.5	0.9
30	1.0	0.6
60	0.37	0.22
120	0.07	0.04
240	0.01	0.006
360	0.0	0.0

Levels of CT989 in tissues / other fluids

SAMPLE	TIME [HRS]	VOLUME [ML]	WEIGHT [g]	AMOUNT [μ g]
Urine	4.5	6	-	198.0
Urine	6	9	-	46.8
Bile	6	1	-	61.3
Kidney	6	-	21.85	210.0
Lung	6	-	23.75	178.0
Liver	6	-	-	negligible

Rabbits were cannulated *via* the femoral vein before the administration of CT435 (2mg/kg) and CT989 (10 mg/kg). Blood samples were then removed directly into ethyl acetate at times ranging from 0 - 6 hrs. The concentration of inhibitor in the blood was then determined by activity assay.

The resulting concentration-time profiles are presented in Fig. 7.4. The elimination kinetics can be described by a 2-compartmental model. Both inhibitors were removed rapidly from the circulation with a $t_{1/2}$ (α) of the order of 1 minute. The $t_{1/2}$ (β) for CT435 was estimated to be 15 minutes and that for CT989 to be 21.6 minutes. After 5 minutes the concentration of CT989 had decreased to 5% of that measured at the first time-point (0.45 mins) (Fig. 7.3). CT435 was also significantly cleared from the blood compartment after 5 minutes. No CT989 was detectable in the blood after 6 hours.

The results indicate that the P_1' isobutyl derivative, CT435, and the P_1' toluylpropyl derivative, CT989, both undergo rapid clearance in the rabbit. The half-lives of both compounds were similar to those of CT1166 and CT1509 in the mouse. CT989 displayed a typical bi-exponential profile in the rabbit in contrast to its behaviour in the mouse (section 7.2:2 {i}).

The CT989 study was expanded to include the measurement of inhibitor concentrations in urine, bile, liver, lung and kidney. The compound was extracted using ethyl acetate directly from the fluids and from homogenised tissues. CT989 activity was quantitated by titration against gelatinase-A. The results of this study are presented in Table 7.4 together with the corresponding blood levels. CT989 activity was detected in all extracts except for liver. The unexpected presence of CT989 in the lungs may arise from the precipitation of this very hydrophobic compound within their network of small blood vessels. The total amount of inhibitor recovered, however, represented only 3% of the administered dose. CE analysis of selected samples identified only intact compound, but this may be due to the extraction procedure which would discriminate against hydrophilic metabolites. Acidification of the urine followed by β-glucuronidase treatment to extract hydrophilic glucuronides failed to account for any more inhibitor.

Subsequent studies using radiolabelled CT1746 have indicated that a high percentage of the inhibitor may have re-entered the gastrointestinal tract in the form of a glucuronidated metabolite (Dr. O. Epemolu, personal communication).

7.2:3 The oral bioavailablity of MMP inhibitors

(i) Screening by pleurai cavity assay

The pleural cavity assay is used to screen for orally active inhibitors. Preliminary experiments showed a positive correlation between water solubility and the oral bioavailability of inhibitors in mice. This is illustrated in Table 7.5 which shows the SAR data for a series of P_2' cyclohexylalanine inhibitors with modifications at the P_3' position (Dr. S. Chander, personal communication). Hence, CT1509 which has an extended hydrophilic morpholino group at the P_3', produced far higher levels of inhibition in this assay after oral dosing than the much more hydrophobic inhibitors, CT1166 and CT989. In order to correlate results in the pleural cavity assay with blood levels (and subsequently bioavailability), the blood concentration of CT1166 and its percentage inhibition (pleural cavity) were determined simultaneously at several time-points. The results of this study demonstrated that inhibition was effectively stoichiometric and that an inhibitor concentration of 20 nM could completely block enzyme action (Fig. 7.4). This narrow concentration range severely limits the information that this assay can provide. Consequently, even though CT1509 appears to be very effective (55% inhibition) one hour after oral administration (Table 7.5), its bioavailability is probably well below 1%.

Despite its limitations, SAR data provided by this assay lead to the important discovery that replacement of the P_2' cyclohexylalanine with tertiary butylglycine could significantly increase oral bioavailability. As a result, the bioavailabllities of three hydroxamic acid inhibitors from the tertiary butylglycine series were determined in mice and rats.

(ii) Oral bioavailability of MPis in mice

Inhibitor efficacy is routinely tested in mouse models of tumour growth and metastasis. In order to aid in interpreting the efficacy data, blood levels were measured in mice after i.v. and p.o. dosing. CT1847 and CT1746 were compared to Ro31-9790. The structures of these compounds are presented in Chapter 5 (Fig. 5.1).

Ro31-9790, CT1847 and CT1746 were administered either intravenously or orally at 10 mg/kg to groups of 5 mice. At various times after administration the animals were sacrificed and samples of blood were removed. Inhibitor was

Figure 7.4

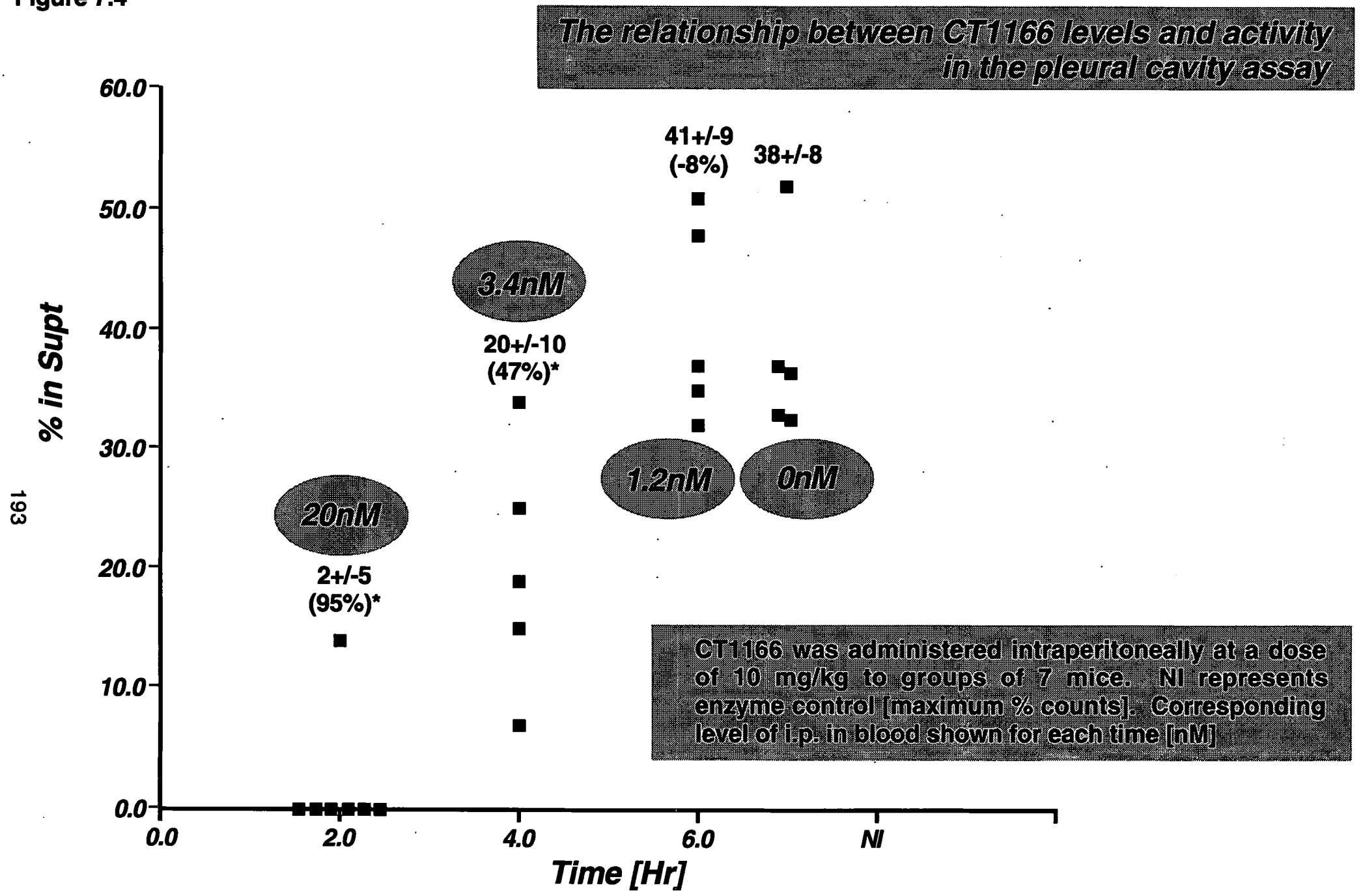

The relationship between CT1166 levels and activity in the pleural cavity assay
60.0
50.0
40.0
30.0
20.0
10.0
0.0
% in Supt
41+/-9
(-8%)
38+/-8
3.4nM
20+/-10
(47%)*
1.2nM
0nM
20nM
2+/-5
(95%)*
0.0
2.0
4.0
6.0
NI
Time [Hr]
CT1166 was administered intraperitoneally at a dose of 10 mg/kg to groups of 7 mice. NI represents enzyme control [maximum % counts]. Corresponding level of i.p. in blood shown for each time [nM]

Table 7.5

Pleural cavity assay of orally administered gelatinase inhibitors

P_3' group	Compound	% Inhibition
Carboxylic acid	CT1508	0
Sulphamyl urea	CT1166	5
Arylsulphonamide	CT989	13
	CT1398	19
Imidazole	CT1400	23
Morpholine amide	CT1575	26
Dimethyl amine	CT1514	40
Morpholine	CT1509	55
Extended chain	CT1495	58

Compounds (80 mg/kg) were administered by oral gavage to groups of 7 mice. After 1 hour assays were initiated by the co-injection of gelatinase-A and [^{14}C]-gelatin into the pleural cavity (Dr. S.K.Chander). Samples were then processed as described in Chapter 2. Results are expressed as a percentage of the gelatinase-A activity obtained in the absence of inhibitor.

extracted from the blood using methanol precipitation and then inhibitor concentrations were determined by titration against gelatinase-A. Concentration-time plots are presented in Fig. 7.5. and pharmacokinetic parameters are shown in Table 7.6.

The systemic half-lives were established from the i.v. traces using a two-compartmental model of drug kinetics (section 2.4:4). In all cases, drug was distributed less rapidly than has been previously described for P_2' cyclohexylalanine inhibitors (section 7.2:2). This is reflected in $t_{1/2}$'s (α) of the order of 3 minutes. After 5 minutes the concentrations of CT1746, CT1847 and Ro31-9790 represented 32%, 19.5% and 17% respectively, of the corresponding 10 second concentrations.

In each case, inhibitor could be detected in the blood 4 hours after administration. The $t_{1/2}$ (β) values were 38, 34 and 36 minutes for CT1746, CT1847 and Ro31-9790, respectively. This value represents a two-fold increase over comparable cyclohexylalanine inhibitors.

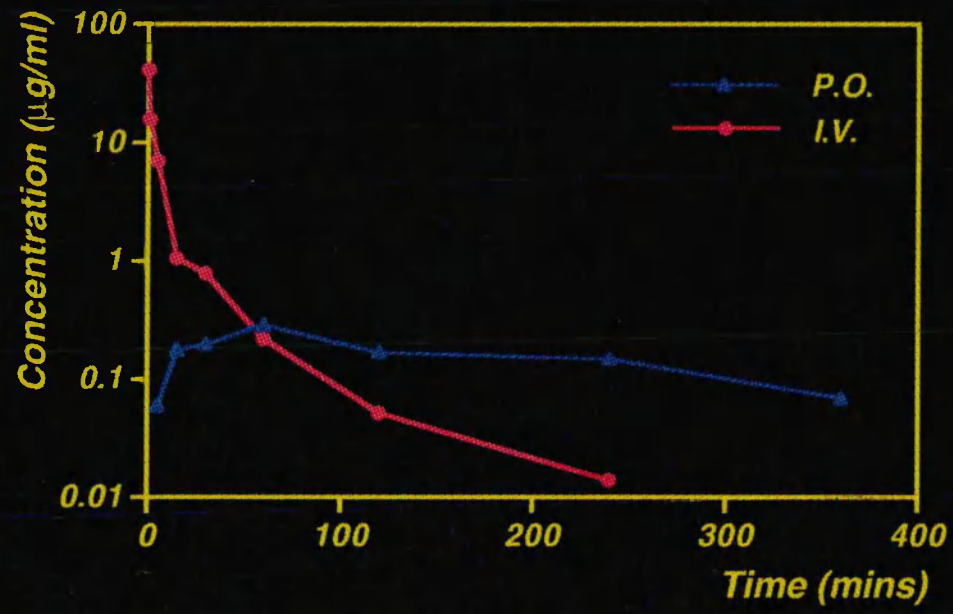
The most striking changes were observed after oral dosing. An oral dose of 80 mg/kg of cyclohexylalanine inhibitors such as CT989, CT1166 and CT1509 produced negligible activity in the blood (data not shown). Ro31-9790, CT1847 and CT1746, however, all remained in the blood for as long as eight hours after a single dose of 10 mg/kg. Activity peaked between 30 minutes (CT1847) and two hours (CT1746) and this was followed by a gradual decrease in inhibitor levels over the following 6 - 8 hours. Areas under the curves were calculated using the trapezoidal method to the last measurable time-point since the flat curve shape does not permit extrapolation to infinity. Oral bioavailability ranged from 35% to 45%. Not surprisingly, the intravenous and oral AUCs were similar for the two most closely related inhibitors CT1847 and Ro31-9790. CT1746, however, produced a larger area under the curve and a higher C_o concentration which indicates a lower volume of distribution (V_D). This was confirmed using the Siphar pharmacokinetic programme. The V_D for CT1746, in particular, indicates the absence of any extensive tissue / protein binding.

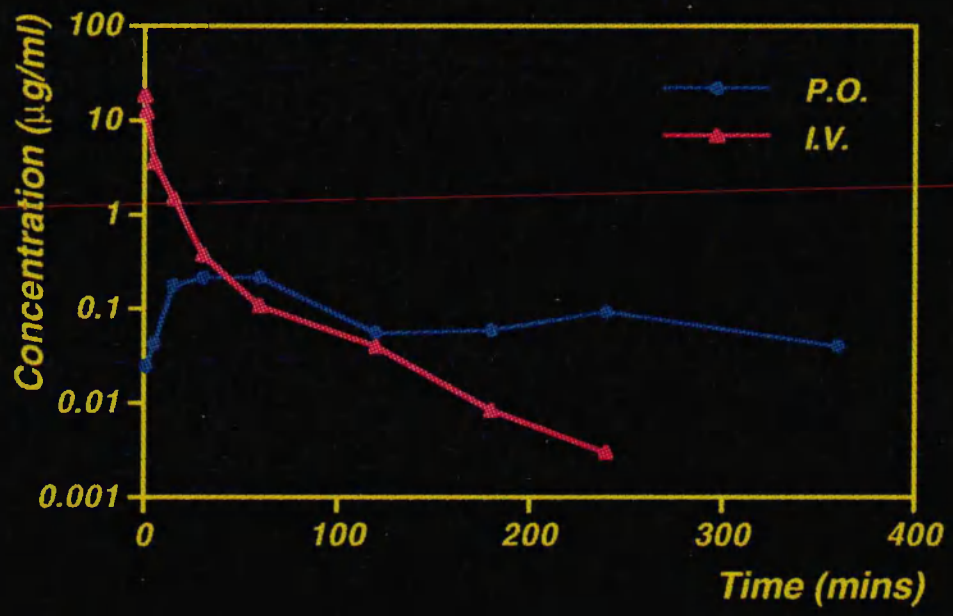
In summary, the replacement of the P_2' cycohexylalanine with tertiary butylglycine significantly increases both plasma half-life and oral bioavailability in mice.

Figure 7.5 Concentration-time piots of 3 MPIs in the mouse.

Ro31-9790, CT1847 and CT1746 (10mg/kg) were administered either intravenously (tail vein) or by oral gavage to groups of 5 mice. At times ranging from 10 secs to 4 hrs (intravenous) and 5 mins to 8 hrs (oral) animals were sacrificed and samples of blood were removed. Inhibitor was extracted from the plasma fraction using methanol precipitation. Blood concentrations were determined by titration of appropriately diluted extract against gelatinase-A. Each point represents the mean of the determinations from 5 animals. (The corresponding pharmacokinetic parameters are presented in Table7.6).

Figure 7.5 Concentration-time plots of 3 MPI's in the mouse





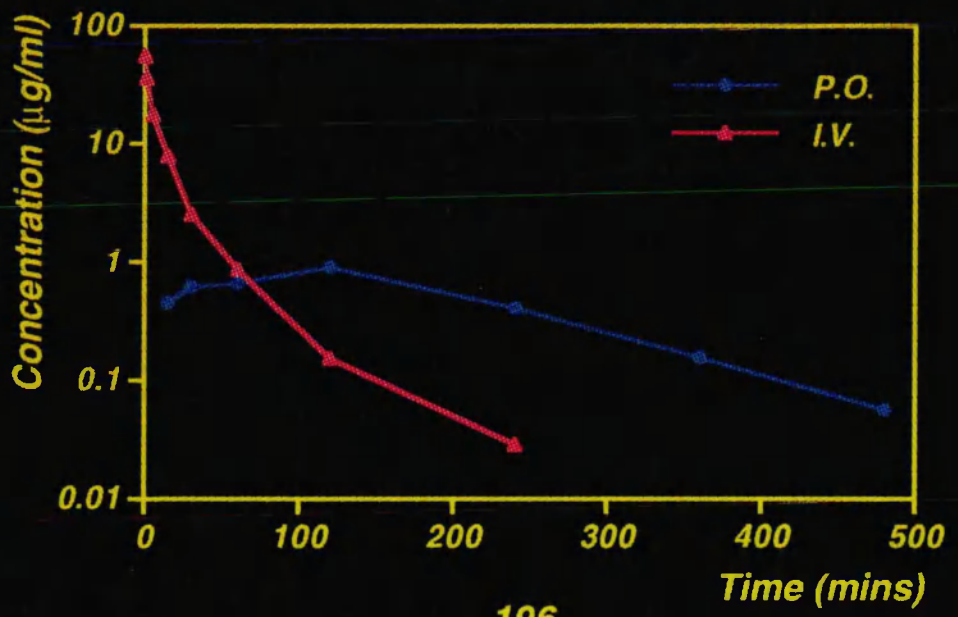


Table 7.6

The pharmacokinetics of 3 MPIs in the mouse

Compound	Terminal half-life (mins)	% Oral availability	V_D (β) (ml/kg)	Total Clearance (ml/kg/min)	MRT (mins)
Ro31-9790	**36**	**36**	**3244**	**62.6**	**16.2**
CT1847	**34**	**34.7**	**4814**	**98.8**	**14**
CT1746	**38**	**44.9**	**1207.5**	**21.9**	**19.3**

Pharmacokinetic parameters were calculated by the Siphar programme using a structurally independent model. Percentage oral availability was calculated from the oral and intravenous AUCs (determined using the trapezoidal rule). The AUC after oral administration was calculated to the final time-point.

Abbreviations :

V_D : volume of distribution

MRT : mean residence time

(iii) Bioavailability and systemic half-lives of CT1746 and Ro31-9790 in rats

The pharmacokinetics of CT1746 and Ro31-9790 were investigated in the rat for 2 main reasons,

1/ This species is used for toxicological studies.

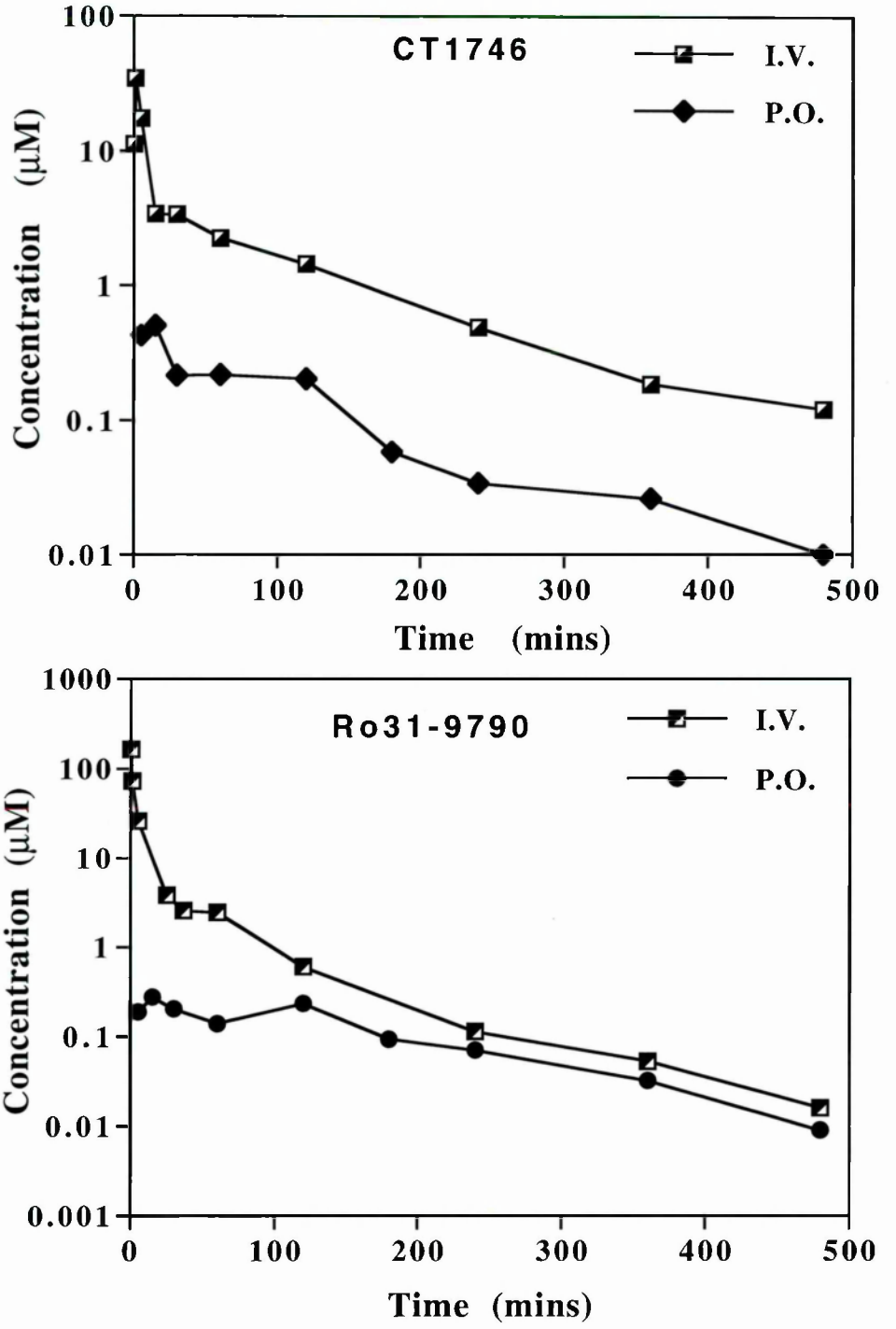
2/ All published data for comparable compounds relates to rat studies.

Dosing regimens and experimental procedures were essentially as described for mice (see above). The resulting plasma concentration-time profiles are shown in Fig. 7.6.

After intravenous administration the activity of both compounds decreased rapidly over the first 15 - 30 minutes and this was followed by a comparably shallow secondary phase which is presumed to represent β-phase elimination. In contrast to the mouse data, the area under the i.v. curve was similar for both compounds producing V_D values intermediate to those calculated for these compounds in the mouse (data not shown). In both cases, inhibitor related activity was detected in the blood 8 hours after administration. Half-lives of 55 minutes and 81 minutes were determined for Ro31-9790 and CT1746, respectively. These values are somewhat higher than those observed in the mouse.

The oral profiles are characteristic of drugs that are either poorly absorbed or suffer extensive first pass metabolism. At no point does the concentration of inhibitor in the plasma overlap the intravenous profile. The maximal blood concentration (C_{max}) occurs 15 minutes after administration and this is followed by a gradual reduction in inhibitor levels that parallels the β-phase elimination observed after i.v. dosing. This indicates that compounds are more rapidly absorbed in the rat than the mouse which exhibits a prolonged absorption phase. The oral bioavailabilities of Ro31-9790 and CT1746 were 5.4% and 7.2%, respectively. The Ro31-9790 result, confirms the low oral bioavailability figure (7%) previously reported by Roche.

Figure 7.6 Concentration-time plots of Ro31-9790 and CT1746 in the rat



Ro31-9790 and CT1746 (10mg/kg) were administered either intravenously (i.v.) or by oral gavage (p.o.) to groups of 5 rats. At times ranging from 10 secs to 8 hrs, 0.5 ml of blood was removed. Inhibitor concentrations were determined as described in Fig. 7.5.

7.3: Discussion

The stabilities of hydroxamic inhibitors were examined in the blood of mouse, rabbit and human. Rat studies were also conducted in collaboration with Dr. G. Curtiss. These *ex vivo* studies provided stability data to augment subsequent *in vivo* investigations. Additionally, this work allowed analytical methods to be tested prior to their use in pharmacokinetic studies.

Pharmacokinetic studies were performed in mice, rats and rabbits to determine the systemic properties of hydroxamic acid inhibitors after intravenous (i.v.) and oral (p.o.) administration. The results of these studies will be discussed in the light of recent data provided by radiolabelled CT1746.

Stability in blood

Blood stability was tested either indirectly by titration against gelatinase-A or directly by chromatographic and electrophoretic methods. The advantage of the former method resides in its sensitivity but the potential for interference by active metabolites is a major disadvantage. Although the low absorbance of these compounds limited the application of techniques which rely on UV detection, such techniques were employed wherever possible so that parent compound could be measured unequivocally.

Prior to analysis, compounds were isolated from contaminating substances using either organic extraction, solid phase extraction or methanol precipitation. Extractions produced more homogenous sample making them more amenable to CE or HPLC. Methanol precipitation, on the other hand is non-selective, which is a major advantage for detecting metabolites.

Hydroxamic acid inhibitors representing the two major classes of compound (P_1' isobutyl or arylpropyl) were tested in the whole blood of several species namely;

(1) Mouse (Tumour growth and metastasis model, pleural cavity model)

(2) Rabbit (Sequential blood samples can be collected from a single animal)

(3) Human (Target species)

In addition, rat studies (Toxicokinetic species) were undertaken in collaboration with an external metabolism group. (Dr. G. Gurtiss, Biodynamics, Cardiff Medicentre).

Results presented in sections 7.2:1 (i) - (iii) and data not shown, demonstrate that the blood stability of these inhibitors follows the general pattern of human > mouse = rabbit > rat. It should be emphasised, however, that no single structure was tested in all the species although the hydroxamic acid was common to all. There was evidence of inhibitor loss in both mouse and rabbit blood after 1 hour. The stability of CT1746 in the plasma of this species (Dr. O. Epemolu, personal communication) suggests that this activity is cell associated. The disappearance of hydroxamates in rat blood is presumably a consequence of high levels of C1 esterase activity. The esterase converts hydroxamate to the corresponding carboxylic acid, a process which has been described for 5-lipoxygenase inhibitors (Summers *et al.*, 1987; Tateson *et al.*, 1988). In fact, degradation is considerably reduced by the addition of an esterase inhibitor (data not shown). From these studies we can predict that MMP inhibitors would undergo extensive metabolism in rats but would be more stable in mice, rabbits and humans. The stability of such compounds in human blood (7.2.1 {ii}) is encouraging for future clinical applications.

***In vivo* studies**

The *in vivo* properties of these drugs have been tested in two ways. Firstly, an *in vivo* gelatinase screen was established. In effect, the *in vitro* [^{14}C]-labelled gelatin assay was transferred *in vivo* by using the mouse pleural cavity to compartmentalise the reagents within the body. Variation of the route, time or concentration of the dose permitted the construction of inhibitor SARs. As an enzyme based assay, however, it can not easily isolate pharmacokinetic and enzymic effects and moreover, its narrow concentration range limits its usefulness. The blood levels of active compounds were, therefore, measured by more quantitative techniques. In one example the contribution of P_3' arylsulphonamide to the pharmacokinetics of the CT989 series was tested after intravenous administration. The results show that arylsulphonamides exhibit distinctive pharmacokinetics in the mouse. After an initial rapid distribution phase compounds appeared to re-enter the circulation with subsequent levels remaining constant for a further several hours. Other inhibitors exhibited a more classical bi-exponential elimination with relatively short β half-lives (~ 20 minutes). These latter results are comparable to those reported by Singh *et al.* (1993) for a range of collagenase inhibitors. The retention of CT989 in the blood

was not detected by the pleural cavity assay which requires freely diffusable inhibitor. This suggests that ethyl acetate may extract the compound from a non-bioavailable pool. A precedent has, indeed, been set by other sulphonamide drugs which are retained in erythrocytes as a result of their interaction with carbonic anhydrase (Gupta, 1987). Comparison of CT989 with CT435 in the rabbit indicated that the unusual mouse profile may be an isolated case. The compounds exhibited similar pharmacokinetics after intravenous dosing.

The development of a CE assay permitted inactive compounds to be tested. Such studies can increase our understanding of how individual chemical features might contribute to the biological profile of a compound by isolating its pharmacokinetic and inhibitory activity. The hydroxamic acid was predicted to be the most pharmacologically active group. Unexpectedly, CT1466 which lacks this group was shown to exhibit similar pharmacokinetics to its parent compound, CT989. Thus a combination of the arylsulphonamide and the overall hydrophobicity of these compounds may overide the influence of the hydroxamic acid. That the arylsulphonamide series may be anomalous in this respect has subsequently been shown using [^{14}C] - radiolabelled CT1746. Metabolism was limited exclusively to the hydroxamic acid which was converted to the carboxylic acid, amide and conjugated glucuronide in both mouse and rat (Dr. Epemolu, personal communication).

The rapid clearance of CT989 from rabbit blood after intravenous administration prompted a limited biodistribution study. Inhibitory activity, presumably due the parent drug, was identified in urine, bile, lung and kidney but not the liver. The presence of both intact drug and metabolites in the bile of rats has previously been described (Singh *et al.*, 1993 Yachetti *et al.* 1993). This would not appear, therefore, to be an uncommon route of excretion for this class of compound. The appearance of CT989 in the kidney and urine indicates an additional excretory pathway although its appearance at this site may be a consequence of kidney damage by precipitated drug. This may also account for the presence of compound in the lung which is the first organ encountered after administration of a drug *via* the femoral vein. The total recovery of drug was estimated to represent only 3% of the administered dose. This indicates that the drug is deposited in tissues other than those described above. Recent studies, using radiolabelled CT1746 have shown that the majority of i.v. administered

compound may be glucuronidated before being emptied into the gastro-intestinal tract (Dr. Epelomu, personal communication).

Oral bioavailability was first tested using the pleural cavity assay. SARs generated by this method identified a requirement for some hydrophilicity, whilst a nitrogen atom was preferred at the P_3' (Table 7.5). The most significant modification, however, proved to be the replacement of P_2' cyclohexylalanine with t-butylglycine. This group has also been used successfully by Roche U.K. in the development of their collagenase inhibitor, Ro31-9790. The bioavailability and pharmacokinetics of such inhibitors were tested in mice and rats. CT1746, CT1847 and Ro31-9790 all exhibited similar concentration-time profiles after i.v. and p.o. administration to mice. Half-lives (i.v.) were in the range of 34 - 38 minutes which represents a two-fold improvement over comparable cyclohexylalanine inhibitors. Percentage oral bioavailability was typically between 35 and 45%, three orders of magnitude improvement over most cyclohexylalanine compounds. The overriding influence of the t-butylglycine is apparent from the similarity of the *in vivo* profiles produced by CT1746, CT1847 and Ro31-9790.

CT1746 was also compared to Ro31-9790 in the rat. The i.v. profiles showed a rapid distribution of both compounds which was followed by a shallow extended β elimination phase. The corresponding half-lives were 55 minutes and 81 minutes for Ro31-9790 and CT1746, respectively. These are longer than would be predicted from the poor stability of these compounds in rat blood. This suggests that the liver plays a more important role in their clearance than blood borne esterases. Extensive liver metabolism is also indicated by the poor oral bioavailability (5 - 7%) of both compounds in this species. Rat gut perfusion studies have shown that CT1746 is efficiently absorbed without modification (Dr. G. Curtiss, personal communication) and therefore the low figure for oral bioavailability is a consequence of first pass metabolism.

In summary, the development of methods for measuring inhibitors in biological fluids permits the stability and pharmacokinetics of these compounds to be investigated. The subsequent generation of biological structure-activity relationships, in turn, allows the identification of chemical features that are responsible for these properties. Inhibitors possessing P_2' cyclohexylalanine have relatively poor *in vivo* characteristics in mice and rabbits. Half-lives are of the order of 15 - 20 minutes and oral bioavailability is negligible. Replacement of

this group with a t-butylglycine produces a doubling in the systemic half-life and significant improvements in bioavailability. Removal of the P_3' group allowed the large aryl group to be retained at the P_1' of CT1746. A combination of these features produced orally active compounds that are potent and selective inhibitors of gelatinase.

Chapter 8

CHAPTER 8
DISCUSSION

The first part of this thesis (Chapters 3 and 4) examines mechanisms involved in the activation of three MMPs, gelatinase-A, gelatinase-B and fibroblast collagenase. This process has been investigated by a number of groups but such studies have often been limited by the small amounts of partially processed enzyme that are available from natural sources. Access to milligram quantities of purified proenzyme through recombinant protein technology and the subsequent development of sensitive and easily manipulated assay systems has allowed their biochemical properties to be examined in more detail. The second part of this thesis describes the use of kinetic assays to identify potent selective inhibitors of gelatinase-A. These studies are extended in Chapter 7 to the measurement of their pharmacokinetic properties and to the subsequent identification of potential clinical candidates.

Since each chapter contains a detailed discussion of the relevant findings, this discussion will be limited to a general summary of the results. It also provides an opportunity to comment on the broader implications highlighted by this work.

Matrix metalloproteinase activation

The over-expression of an enzyme may be used as an indication of its role in disease. That stromelysin and collagenase contribute to the outcome of joint inflammation may, for example, be suspected because their levels are significantly increased by inflammatory mediators. The evidence for elevated levels of progelatinase-A at sites of tumour invasion is less clear. In fact, there is some experimental evidence to support the proposal that invasion correlates, not so much with increased expression of progelatinase-A but with an increase in the proportion of the activated form (Dr. T. Baker, personal communication). The activation of gelatinase-A is, therefore, of considerable interest and the mechanisms by which it is achieved are only now being elucidated. In this context, the recent description of a potential cellular activator (Sato *et al.*, 1994) is of significant interest. Chapter 3 reports the findings of a study on the activation of progelatinase-A by APMA and proteinases. Although this work was aimed towards establishing mechanisms of *in vitro* activation, the results of these

studies may contribute to our understanding of the activation of progelatinase-A *in vivo*.

APMA induction of progelatinase-A self-cleavage results in the removal of the 80 amino acid propeptide to produce a single, active form with an N-terminus Y^{81} (Stetler-Stevenson *et al.*, 1989). The steps involved in this activation process were investigated using inhibitors and by measuring its dependence on enzyme concentration to discriminate between intra- and intermolecular events. The reaction proceeds *via* a transient intermediate which proved to be too unstable for N-terminal sequencing. By homology with stromelysin (Okada *et al.*, 1988) this intermediate is probably the product of a cleavage that occurs a few residues upstream of the conserved propeptide cysteine. That the first step is intramolecular was shown by the fact that the rate of propeptide cleavage was not dependent on the enzyme starting concentration and by the inability of inhibitors to halt it. Presumably, this region of the propeptide lies within the substrate binding cleft and it is cleaved when the displacement of the cysteine from the catalytic zinc by APMA, allows the entry of water to render the enzyme catalytically competent. The remainder of the propeptide is then rapidly removed to produce the mature enzyme. The concentration dependence of this second cleavage and its sensitivity to inhibitors establishes that this is an intermolecular event.

The action of APMA is presumed to be artificial because a physiological counterpart has not yet been identified. A possible candidate for this role was proposed by Weiss *et al.* (1983). Endothelial cell-stimulating angiogenesis factor (ESAF) is a low molecular mass product of cloned lymphoma cells which activates fibroblast procollagenase and possibly progelatinase-A. Interestingly, its activity is enhanced by heparin which has also been shown to increase the rate of gelatinase-A self-activation (Crabbe *et al.*, 1993). Presumably, the mechanism behind the two processes is similar and results from the ability of heparin to concentrate the reactants on its surface.

In vivo it is probable that MMP activation is initiated by other proteinases which are capable of removing the first 30 to 40 amino acids of the propeptide (Nagasi *et al.*, 1991). Cleavage occurs at a "bait region", the sequence of which determines the class of endopeptidase capable of activating the MMP in question. Progelatinase-A, however, has proved resistant to *in vitro* activation by either serine proteinases or stromelysin (Okada *et al.*, 1990). This can be linked

to the absence of a susceptible peptide bond in the bait region. The discovery that progelatinase-A could be activated by cell membranes in the absence of an exogenous activator (discussed in Chapter 3), however, indicated that the cell may provide a component which is missing from *in vitro* reactions. It can be argued that plasmin may be able to activate progelatinase-A in the presence of this cellular component and that this explains why inhibitors of urokinase can block the type IV collagenase activity of metastatic cells (Reich *et al.*, 1988). The identity of such a component has recently been reported by Sato *et al.* (1994) although it appears to activate progelatinase-A directly, rather than by a plasmin cascade. Given the ability of the TIMPs to block cell surface activation (Ward *et al.*, 1991) the activator has, not surprisingly, been shown to be a new member of the MMP family, namely, membrane type matrix metalloproteinase (MT-MMP). It binds to the plasma membrane by virtue of a lipophilic anchor-region situated within its C-terminal domain. MT-MMP contains a sequence at the end of its propeptide, which is also present in stromelysin-3, that is known to be cleaved by a group of subtilisin-like processing enzymes. Since these enzymes are located in the golgi (Misumi *et al.*, 1991), MT-MMP may already be active before it is transported to the cell surface.

Work presented in this thesis shows that progelatinase-A can also be activated by comparatively high concentrations of soluble MMPs, namely matrilysin and interstitial collagenase. The ability of collagenase to process progelatinase-A can be significantly enhanced in the presence of heparin, which also stimulates gelatinase auto-activation (Crabbe *et al.*, 1993). That this activity is mediated by the C-terminal domain of gelatinase-A was shown using a deletion mutant (proNGL-A) that lacked this domain. Heparin stimulated activation may not be confined to the test-tube as will be discussed later. Matrilysin can process progelatinase-A to the mature form, although the sequence of events is complicated by the autolytic action of gelatinase-A. Collagenase, on the other hand, cleaves only at N^{37}-L^{38}, which seems to be sufficient to allow gelatinase-A to complete the process. The sequence containing this bond presumably corresponds to the bait region of other MMPs. The same intermediate is produced by the action of stimulated human fibrosarcoma cells on progelatinase-A (Strongin *et al.*, 1993). Significantly, this cell-line was reported to produce elevated levels of both collagenase and matrilysin (Overall and Sodek, 1990). Since matrilysin and collagenase are often

co-expressed in cell-lines their individual contributions to progelatinase-A activation *in vivo* may be difficult to establish. This might be facilitated by the use of specific inhibitors but as shown in Chapter 5, the two enzymes have similar inhibitor selectivities. Moreover, if the cleavage site of MT-MMP proves to be at N^{37}-L^{38}, this enzyme may also possess a collagenase-like specificity. Alignment of its substrate binding sequence with that of other MMPs (Fig. 1.11) argues against this, however, since its S_1' sub-site shows gelatinase-like features.

Although these experiments do not establish a role for any individual MMP in the activation of progelatinase-A they do show that it is likely to be a result of MMP action. It is also possible that the MMP responsible may vary according to the physiological circumstances.

The activation of progelatinase-B has been studied extensively by a number of groups but the data emerging is equivocal. Using progelatinase-B purified from neutrophils, Triebel *et al.* (1992) demonstrated that autoprocessing of the proenzyme in the presence of APMA occurred by a stepwise truncation of the propeptide to M^{75}. At the same time a three step loss of C-terminal domain fragments to A^{506} produced a final species of 73 kDa. The 73 kDa form was reported to exhibit full activity despite retaining the part of the propeptide that contains the cysteine that is thought to bind to the active site zinc atom. The specific activity was not compared, however, with a form processed beyond the cysteine. Ogata *et al.* (1992) showed that stromelysin treatment of progelatinase-B produced an 82 kDa active form with F^{88} at the N-terminus which by homology with other members of the family represents the fully active enzyme. In contrast, Okada *et al.* (1992) reported that stromelysin treatment generates a 64 kDa species. Goldberg *et al.* (1992) generated various C-terminal mutants of progelatinase-B and found that some mutations produced a proenzyme that could not be fully processed by either APMA or stromelysin. They propose that the C-terminal domain may be in some way involved in processing.

Data from a comparative study of trypsin, APMA and stromelysin activation of progelatinase-B are described in Chapter 3. In response to the reports from Goldberg *et al.* (1992), the contribution of the C-terminal domain was also studied using a deletion mutant lacking the entire domain, proNGL-B. The resulting data show that, in the presence of APMA or stromelysin, proNGL-B generates the same amino-terminus as the full-length enzyme i.e. M^{75} and F^{88}, respectively. The inability of APMA to induce a cleavage at F^{88} suggests that

gelatinase-B does not recognise this site. This is somewhat surprising since gelatinase-A, which is reported to have a similar substrate specificity does cleave itself at this position. In agreement with Ogata *et al.* (1992), stromelysin processing of the wild-type enzyme is confined to removal of the propeptide. The 64 kDa species, described by Okada, is formed when stromelysin is added to progelatinase-B that has been stored for prolonged periods. The reasons for this are unclear although collagenase is also known to cleave in its own hinge region more readily after storage (unpublished observation). In contrast, exposure to APMA causes self-processing at both the N-terminal and C-terminal ends of the molecule and the final form is produced at a slower rate than that generated by either stromelysin or trypsin. Self-processing and activation of wild-type and truncated enzymes occurred at a similar rate. Moreover, there were no significant differences in the specific activity of each form against gelatin and a peptide substrate. These data suggest that the C-terminal domain of progelatinase-B does not determine the autoprocessing specificity of the catalytic domain nor the final substrate specificity. The latter observation is consistent with previous observations on the role of the C-terminal domains of gelatinase-A and stromelysin (Murphy *et al.*, 1992a; Murphy *et al.*, 1992b). Surprisingly, the continuing presence of the propeptide cysteine in the APMA generated forms had no effect on the final specific activity, thereby confirming the observations of Triebel *et al.* (1992).

Interstitial procollagenase can be partially activated by APMA and trypsin (Grant *et al.*, 1987; Nagase *et al.*, 1990; Suzuki *et al.*, 1990), however, full activity against collagen requires processing to the F^{81} N-terminus. This is commonly effected by the addition of stromelysin in a process that is called "superactivation". This form of the enzyme is 5 - 8 fold more potent against collagen (Murphy *et al.*, 1987; Suzuki *et al.*, 1990) but its relative specific activity against peptide substrates has not been reported. Reinemer *et al.* (1994) arrived at a hypothesis for the role of F^{81} by examining its position relative to other amino acids in a crystallographic model of the catalytic domain of neutrophil collagenase. They propose that a salt-bridge links the amino-terminal phenylalanine and an aspartic acid (D^{232}) close to the end of the catalytic domain. This interaction results in a structurally more ordered N-terminal fragment which, unlike forms that lack this residue can then no longer block the

substrate binding cleft of an adjacent molecule. Based on this proposal, superactivation should increase the activity of collagenase against peptide substrates in addition to collagen. Data presented in this thesis (Chapter 4) provide no evidence for this proposal. The presence of F^{81} at the N-terminus conferred no additional activity against peptide substrates although removal of the propeptide cysteine appeared to be necessary for full activity. This would suggest that the presence of F^{81} is only required for activity on collagen and that the association with the C-terminal aspartic acid (D^{232}) may have a different function. On inspection, D^{232} is seen to lie close to the hinge region linking the catalytic domain with the C-terminal collagen binding domain. This is particularly short in collagenase and may be important in allowing the C-terminal domain to position the catalytic domain in the precise site for collagen cleavage. The phenylalanine-aspartic acid interaction may function similarly to maintain the correct orientation between the C-terminal domain and the catalytic domain. The proximity of F^{81} and, therefore, the propeptide to the C-terminal domain may also explain the observation that collagenase only binds to collagen in the active form (Vater *et al.*, 1978; 1986; Murphy *et al.*, 1992b). Presumably, this places the propeptide in a position where it can mask the collagen binding exo-site.

The activation of collagenase by stromelysin may be a means of fine tuning its activity *in vivo*, since the expression of both enzymes is significantly elevated by inflammatory mediaters such as IL-1 and TNF and raised levels of both enzymes are observed in joint destruction (Walakovits *et al.*, 1992). The presence of highly sulphated proteoglycans in the joint matrix may make the local environment acidic. Outside this environment stromelysin would be a far less effective activator since it has a pH optimum close to pH 5 (Harrison *et al.*, 1992). Indeed, it retains less than 10% of its activity at pH 7.2 - 7.4 (unpublished observation). At physiological conditions of neutral pH, the role of stromelysin may be taken up by other MMPs such as matrilysin (Quantin *et al.*, 1989).

The possibility that gelatinase-A is also capable of processing collagenase was noted whilst studying the activation of progelatinase-A by collagenase. A reduction in the size of the collagenase correlated with the appearance of active gelatinase-A and N-terminal sequencing of the newly formed collagenase revealed its N-terminus to be F^{81}. The rate at which collagenase was processed was slow compared to processing by stromelysin, even at pH 7.5. The addition of heparin, however, significantly enhanced activation by gelatinase but not

activation by stromelysin. The combination of fully active collagenase and gelatinase is a potent collagen degrading force. These observations show that gelatinase-A and collagenase may be involved in a reciprocated activation process which is enhanced by heparin. This activity may not be limited to the test-tube since all of these agents are be present at sites of mast cell action. Mast cells participate in a variety of inflammatory processes and are often found in the stroma at sites of tissue remodelling. Moreover, mast cells are also a source of tryptase and chymase, proteinases that can activate stromelysin (Gruber *et al.*, 1989) and collagenase (Saarinen *et al.*, 1994; Lees *et al.*, 1994), respectively. The presence of these cells at tumour sites before the ingrowth of new capillary sprouts (Taylor and Folkman, 1982) suggests that they may also play a role in angiogenesis. These authors also show that the release of heparin increases the migration of capillary endothelial cells *in vitro*. The involvement of MMPs in angiogenesis has also been demonstrated by the use of inhibitors to these enzymes (reviewed in Moses and Langer, 1991).

Reciprocated activation may also explain some of the conflicting experimental data that has emerged from studies on the relative contributions of serine proteinases and MMPs to basement membrane invasion. Reich *et al.* (1988), for example show that inhibitors of both collagenase and plasminogen activators block the invasion of an artificial basement membrane by metastatic cells. Reversal of the effects of plasminogen activators by the MMP activator, mersalyl (similar to APMA) lead the authors to propose that a plasmin - gelatinase cascade is responsible for basement membrane degradation. A similar pathway had already been proposed by Mignatti *et al.* (1986) on the basis of their study of proteinases involved in the invasion of amniotic basement membrane by B16 melanoma cells. The results in this thesis support an alternative mechanism in which plasmin activates collagenase, setting in motion the reciprocated activation process described earlier. A summary of possible pathways leading to progelatinase-A and procollagenase activation is presented in Fig. 1.8.

The requirement for an intact C-terminal domain for collagenolytic activity has been demonstrated by several groups (Birkedal-Hansen *et al.*, 1988; Clark and Cawston, 1989; Murphy *et al.*, 1992b). In Chapter 4 the absence of this domain in a deletion mutant was shown to abolish collagen degrading activity without affecting the peptidase activity of collagenase. The function of the C-

Figure 8.1 Zymogen activation cascade responsible for matrix breakdown

Fig. 8 summarises the possible pathways leading to the superactivation of collagenase and the activation of progelatinase-A and hence, matrix breakdown. A hypothetical membrane-dependent cascade is presented in the lower part of the figure. This shows the activation of MT-MMP (Sato *et al.*, 1994) by subtilisin-like enzymes in the Golgi. The active MT-MMP is then inserted into the cell membrane where it can initiate the activation of progelatinase-A.

The upper part of the figure depicts the involvement of plasmin in MMP activation. Procollagenase may be partially activated by plasmin to a species which retains the propeptide cysteine but has an exposed superactivation cleavage site. Collagenase is known to be cleaved at this site (F^{81}) by stromelysin (Murphy *et al.*, 1987).

The present work has demonstrated that superactivation may also result from gelatinase-A action and that this is enhanced in the presence of heparin. This activity is reciprocated because active collagenase can process progelatinase-A to a form which rapidly undergoes autolytic activation. These events may occur at sites of mast cell action since they are a source of heparin, in addition to chymase and tryptase which activate procollagenase (Saarinen *et al.*, 1994) and prostromelysin (Gruber *et al.*, 1989), respectively. Matrilysin may be important in other circumstances, since it can directly activate progelatinase-A and superactivate collagenase (Quantin *et al.*, 1989).

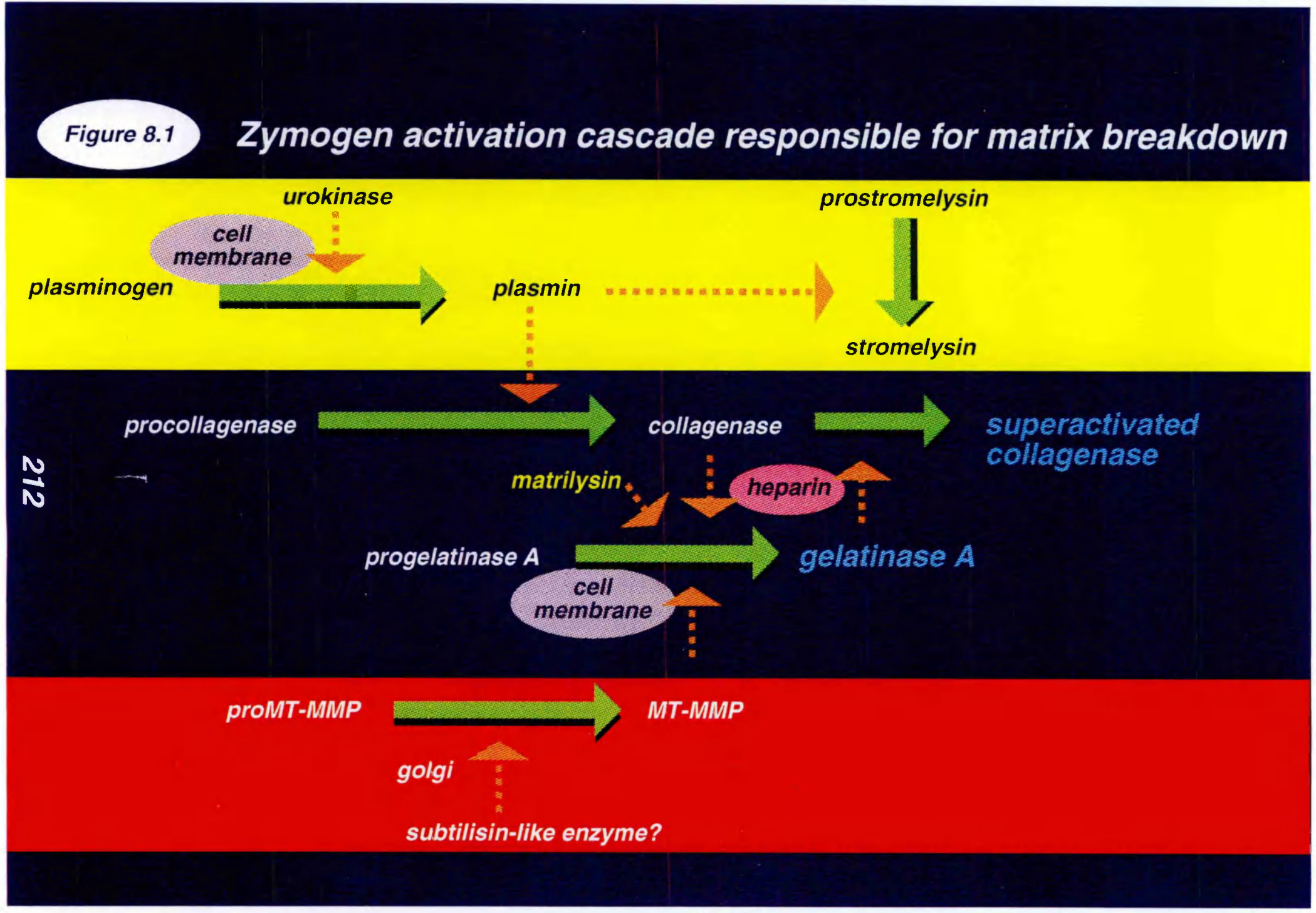


Figure 8.1
Zymogen activation cascade responsible for matrix breakdown
urokinase
cell membrane
plasminogen
plasmin
prostromelysin
stromelysin
procollagenase
collagenase
superactivated collagenase
matrilysin
heparin
progelatinase A
gelatinase A
cell membrane
proMT-MMP
MT-MMP
golgi
subtilisin-like enzyme?

terminal domain in directing substrate cleavage is unique to collagenase. The importance of this domain in collagenolysis makes it a potential target for collagenase inhibitors. This was tested by screening for monoclonal antibodies against collagenase that could block collagenolysis. During the course of this study an antibody (MAC065) was discovered which selectively inhibited collagenolysis with an IC_{50} of 3 nM, without affecting the peptidase activity of collagenase. Since this molecule does not cross-react with other MMPs it may provide an exquisitely selective means of blocking collagenase action. Moreover, this approach may be extended to other MMPs once the role of their potential substrate/matrix binding domains has been established.

The design of matrix metalloproteinase inhibitors

Captopril is widely regarded as the first example of an orally active metalloproteinase inhibitor. The design of this drug advanced the now common principle of combining a zinc chelating group with a pseudo-peptide structure to provide specificity. This principle was subsequently applied to the design of collagenase inhibitors by Gray *et al.* (1981). The resulting compound exhibited a relatively poor IC_{50} of 10 μM against tadpole collagenase. More potent compounds were discovered using SARs so that within 5 years of the Gray structure, several groups were reporting IC_{50}'s for collagenase in the low nanomolar range (Dickens *et al.*, 1986; reviewed in Johnson *et al.*, 1987). The Searle hydroxamic acid based compound described in Chapter 1 (Fig. 1.9) is representative of the structures available at the time. Since then, four major issues have been raised, namely,

1. Which enzyme to target?
2. Is a selective inhibitor preferable to a broad spectrum inhibitor for cancer treatment?
3. How do you obtain selectivity?
4. How can a peptide based inhibitor be converted into an orally active drug?

Most of the early studies were directed against the best characterised MMP of the time, *i.e.* interstitial collagenase. There were good reasons to think that this enzyme was an important target for the treatment of rheumatoid arthritis. Loss of the articulating surface of the joint is the major disabling feature in this disease and collagenase is the only known mammalian enzyme capable of specifically cleaving the bundles of fibrillar collagen which form a large part of the

joint matrix. Consequently, early inhibitors were based on the collagen cleavage site and in keeping with collagenase's specificity for collagen, leucine-like groups were invariably incorporated into the P_1' position.

Attention later turned to the destruction of proteogylcans observed in the traumatised joint (Walacovits *et al.*, 1992). The enzyme responsible for this matrix breakdown was originally identified as proteoglycanase (Galloway *et al.*, 1983) on the basis of this activity but has since been renamed stromelysin, in recognition of its much broader substrate specificity. The cloning and purification of a recombinant form of this enzyme (Whitham *et al.*, 1986; Murphy *et al.*, 1987) greatly facilitated the task of designing more selective inhibitors. Another significant advance was made by the introduction of synthetic fluorescent peptide substrates (Stack and Gray, 1989: Knight *et al.*, 1992). Nonetheless, progress in the design of non-collagenase inhibitors remained slow, largely because of a general reluctance to move away from the use of a leucine-like group in the P_1' position. Indeed, there is little evidence of any significant modifications to this group prior to the studies undertaken at Celltech.

As reported earlier, access to milligram amounts of recombinant enzymes followed by the establishment of easily controlled and sensitive peptide assays were key elements in the progression of inhibitor design at Celltech. The main findings of this work are discussed in detail in Chapter 5 and so will only be covered briefly in this section, together with some observations made during the screening process which have not previously been described.

The first Celltech inhibitors to be tested were similar to the structure of Compound 5 (Fig. 1.9), described by Di Pasquale *et al.* (1986). This had three features which were retained in subsequent lead molecules namely,

1. It is right-hand side inhibitor.
2. It possesses R,S,S stereochemistry.
3. The zinc binding ligand is a hydroxamic acid.

The strategy applied at Cellech was to design selective inhibitors of the target enzyme, initially stromelysin, to minimise the side-effects that might result from modifying normal connective tissue turnover. Several broad range inhibitors were also designed as tools to probe the contribution of individual MMPs to disease progression. The main site of substrate recognition is the S_1' pocket. The realisation that stromelysin self-cleaved at an aromatic residue eventually

led to the introduction of a phenylpropyl group into the corresponding P_1' position of inhibitors. As predicted this fulfilled the dual role of increasing activity against stromelysin whilst discriminating against collagenase. This distinction was maintained when the target was switched to gelatinase-A because of its putative role in the metastasis (Chapter 1). Like stromelysin, gelatinase-A self-cleaves at the amino-terminal side of an aromatic residue. This choice of enzyme target proved to be fortuitous for inhibitor design, since it later became evident that selectivity for gelatinase over collagenase could readily be achieved. The converse is recognised as being far more difficult, an observation which can now be rationalised on inspection of their substrate-binding clefts. Fig. 1.11 shows how enzyme homology can be used to predict which amino acids are involved in forming enzyme binding pockets (Gooley *et al.*, 1994). Gelatinase-A is thought to possess a large S_1' sub-site which can easily accept the leucine preferred by collagenase. In contrast, the smaller S_1' pocket of collagenase would not tolerate the bulky aryl groups favoured by gelatinase-A. Identification of the phenylpropyl P_1' group was a significant step in the effort towards discovering a selective and potent gelatinase inhibitor. This effort was maximised by screening inhibitors against a panel of MMPs, the results of which allows the following conclusions to be drawn. Essentially, gelatinase-A, gelatinase-B and stromelysin appear to have similar recognition sites characterised by a preference for a large P_1' and P_3'/P_4' group. Matrilysin, like collagenase, cannot tolerate such a P_1' group and neither of these enzymes appear to form significant interactions with other inhibitor side-chains. As described above, these conclusions may be rationalised from modelling data based on X-ray crystallography and NMR (discussed in Chapters 1 and 5). In addition to post-rationalisation of SAR data, the work of Gooley *et al.* (1994) shows how inspection of the residues involved in P_1' binding can be used to predict enzyme selectivity. This was tested using neutrophil collagenase for which there is little available SAR data. Surprisingly, since collagen is a major substrate for this enzyme, it possesses an S_1' pocket which bears more resemblance to that of gelatinase-A than to that of interstitial collagenase. On this basis it was predicted that it would accept inhibitors with aryl groups at the P_1' position. This proved to be the case (unpublished observation) and, indeed, aryl based inhibitors may be as potent against this enzyme as gelatinase-A.

Both SAR and structural evidence indicate that the S_2' and S_3' sub-sites are shallow and the corresponding inhibitor side-chains face away from the bulk of the enzyme toward the solvent. Whilst this limits the potency and selectivity that can be incorporated into these positions it does present the opportunity to modify the physiochemical properties of the inhibitor. Incorporation of a biotin into the P_3' position, for example, produced a compound similar to CT733 (Table 5.4) that was subsequently used for localising MMP activity on breast cancer cells (Dr.T.Baker, personal communication). Modifications at the P_2' residue contributed towards oral bioavailability (see later). The general requirements for gelatinase inhibition are summarised in Fig. 5.2.

Given that many different classes of zinc metallopeptidase reside on the extracellular surface of plasma membranes and are, therefore, highly accessible it was important that SARs were not confined to the study of MMPs. It emerged from these studies that aminopeptidase N, neprilysin and a mouse enzyme, meprin, did not accept the extended aryl group at the P_1'. In the case of neprilysin this is unexpected, since inhibitors such as thiorphan (Fig. 1.8) possess a phenylalanine analogue in this position (Roques *et al.*, 1980). The lack of activity against ACE is somewhat less surprising since this enzyme is a carboxypeptidase and interacts with the C-terminal carboxylic acid of its substrates. Moreover, ACE is reported to prefer (S)-stereochemistry at the P_1' chiral centre (reviewed in Johnson *et al.*, 1987).

It is apparent that the kinetic properties of the inhibitors described in this thesis are very similar to those reported for ACE, including Ki's in the picomolar range. In such cases the rate of establishment of the equilibrium between the reactants and the complexes must be slow relative to those involving the substrate. Morrison and Stone (1985) summarised the consequences of this as follows. Low Ki values may derive from a low dissociation rate or a high association rate or both. Since there is an upper diffusion controlled limit of 10^9 $M^{-1}s^{-1}$ for the second order rate constant (k_{on}) and $Ki = k_{off}/k_{on}$, this means that an inhibitor with a Ki of 10^{-12} M will exhibit a dissociation half-time of at least 11.5 minutes (see Table 6.1 for details). Furthermore, the rate of enzyme-inhibitor complex (EI) formation would also be slow since the first order rate constant k_{on}I includes a term for free inhibitor, which in an inhibitor study is varied at concentrations in the region of its Ki value. The effects arising from slow

establishment of equilibria can be avoided by preincubating the inhibitor with enzyme and then performing a steady-state analysis using the general equation for tight-binding and competitive inhibition described by Morrison and Walsh (1988).

From the argument outlined above we can see that such inhibitors may be studied using slow-binding kinetics even if their association rates approach diffusion limits. This is particularly valuable for analysing the individual contributions of k_{on} and k_{off} to the Ki value. Baici (1988) points out that for an inhibitor to be effective it must be able to bind to the target enzyme at a rate greater than that of its natural substrate, otherwise significant substrate turnover would occur before equilibrium of the enzyme-inhibitor complex. MMPs are surrounded by an abundance of substrate which may have a significant effect on the delay time for inhibition. Consequently, the k_{on} value of an inhibitor should ideally be large. Measurement of this value was facilitated by the high turnover rate of QF24, by gelatinase-A. This allows the enzyme concentrations to be kept low so that slow-binding equations can be applied. Methods for determining k_{on} are discussed in Chapter 6. Briefly, this value was determined either indirectly by linear regression of k_{obs} (apparent first order rate constant for establishment of equilibrium) on the inhibitor concentration or directly using λ, a term relating the k_{on} to the total inhibitor and enzyme concentration. The linearity of the former plot can be interpreted as an indication that enzyme-inhibitor complexes arise from a simple bimolecular collision. In most instances k_{on} was above the limit of $10^6\ M^{-1}s^{-1}$ proposed by Bartlett and Marlow, (1987) for slow binding inhibition. The broad range inhibitor, CT435 (Table 5.2), proved to be an exception, although its association rate constant was not significantly below the threshold cited above. Dissociation half-times, on the other hand, were consistently of the order of minutes to hours (Table 6.1). This shows that slow rates of dissociation are largely responsible for the slow establishment of equilibrium and a combination of this and a relatively rapid association rate result in inhibitors with Ki's in the picomolar range. The studies provide some indication of the relationship between inhibitor structure and association and dissociation rates. Surprisingly, both features appear to correlate with the size of the P_3' group. An increase in k_{on}, for example, was observed upon replacement of this group with an amide (CT1746), whereupon, incorporation of a large group in this position eg. sulphamylurea (CT1166) or arylsulphonamide (CT989) significantly

decreased the rate of dissociation resulting in inhibitors with Ki's of less than 10 pM against gelatinase-A. In another example, extension of the P_3' chain of CT543 by a single carbon (CT962) resulted in a ten fold decrease in the dissociation rate. Other groups may, however, make a significant contribution. Given that association rates are generally of the same order it is probable that any modification leading to a significant decrease in potency will be reflected in a higher k_{off} value. Replacement of the hydroxamic acid of CT989 with a carboxylic acid (CT976), for example, leads to a thirty fold increase in k_{off} (Fig. 6.2). Obviously, the small sample size of this study precludes a more detailed analysis of the relationship between inhibitor structure and rates of association and dissociation with gelatinase-A.

There are comparatively few peptide based drugs in the clinic and this may be a consequence of their poor pharmacokinetic properties (Humphrey and Ringrose, 1986). That this is the case in the MMP area is reflected in the paucity of literature on the subject. Despite this obstacle, oral availability was a major goal in the design of the MMP inhibitors described in this thesis.

The published results may be summarised as follows (mainly from Singh *et al.*, 1993). The sytemic half-life in rats is generally of the order of 6 - 40 minutes. There are no reports of oral bioavailabilities greater than 10% and in most cases it appears to be negligible. Compounds appear to be subject to considerable first pass elimination. Analysis of the bile has identified the presence of parent compound and metabolites arising from modifications of the hydroxamic acid. Roche report a systemic half-life of about 1 hour in the rat and bioavailabilities of 7% and 17% in the rat and marmoset, respectively for Ro31-9790, which incorporates a tertiary butyl group in the P_2' position. This is a broad spectrum MMP inhibitor, which is currently being developed for the treatment of arthritis.

British Biotech have adopted a different strategy in which the hydrophobic properties of their lead compound (BB94;Batimastat) have been maintained. Not unexpectedly, this makes the drug orally non-bioavailable but it imparts a long lasting depot effect when administered intraperitoneally. This formulation is reported to show beneficial effects in resolving tumour ascites (Beattie *et al.*, 1994).

As a consequence of the specificity of the enzyme-inhibitor interaction a considerable reduction in potency may be expected after the incorporation of features aimed at improving bioavailability. The Celltech strategy is to develop inhibitors efficient enough to withstand this predicted drop in potency without significantly modifying their ability to target the enzyme. The development of methods to measure the activity of these inhibitors *in vivo* has been a key element in the progression of this work.

Two major questions were addressed,

1. How to rapidly screen inhibitors for improved pharmacokinetic properties?
2. How to measure the plasma levels of compounds that possess very poor chromophores?

The first issue was overcome by the development of the pleural cavity assay. This screen is based on a model described by Lark *et al.* (1990). In that model, stromelysin and radiolabelled transferrin were injected into the pleural cavity of rats and the effect of TIMP-1 administration was observed by analysing the contents of the pleural cavity. Transferrin is a very poor substrate for gelatinase-A (unpublished observation) and so it was substituted with radiolabelled gelatin. The assay was transferred to the mouse since efficacy models were established in this species. This assay was used to screen a number of inhibitors, initially following intravenous (i.v.) or intraperitoneal (i.p.) administration. Activity after i.v. dosing tended to correlate with *in vitro* potency, implying that inhibitors had very similar systemic properties. Particularly hydrophobic compounds such as CT989 exhibited more prolonged activity after i.p. administration, which indicates that this compartment can be used to provide a slow release mechanism. Similar properties were described for Batimastat (see above).

When studies were extended to the oral route none of these early compounds exhibited significant bioavailability. Results in Chapter 7 show that small improvements in oral bioavailability were effected by the introduction of a basic group into the P_3' position (Fig. 7.5). The subsequent incorporation of a tertiary butylglycine group in the P_2' position led to a dramatic improvement when it was combined with the removal of the P_3' residue. Retention of the aryl P_1' group meant that such inhibitors remained selective against collagenase and retained a significant level of potency (20 - 40 pM) for gelatinase-A.

The pleural cavity assay was useful for providing SAR data but it could not be applied to the determination of pharmacokinetic properties. Accordingly, a range of extraction and detection methods were developed to determine the concentration of inhibitors in biological fluids. These were tested initially in a limited study of the stability of lead compounds in blood or plasma. The results indicate that inhibitors may be unstable in rodent blood although there is only evidence of serious instability in rat blood (Dr. G. Curtiss, personal communication). The good stability of a prototype compound, CT420, in human blood has since been established for other compounds (unpublished observation).

Methods developed for measuring blood stability were subsequently applied to pharmacokinetic studies. Titration of the inhibitory activity was generally employed for this purpose after an appropriate extraction step. One drawback is the inability of this method to discriminate between parent and metabolites of similar activity. Since, however, the hydroxamic acid is predicted to be the major site of metabolism, the major metabolites are not expected to be particularly active. Nonetheless, wherever possible analytical techniques were used to confirm the data and to identify the parent compound.

Early studies identified the arylsulphonamide series as having superior systemic properties in the mouse. Further investigation indicated that the hydroxamic acid was not the major determinant of their clearance. The atypical blood profile seen in the mouse was not displayed in the rabbit. Analysis of rabbit bile and organs associated with elimination accounted for only 3% of the administered dose. Other studies suggest that a large proportion of this dose may enter the GI tract in the glucuronide form (Dr. O. Epomolu, personal communication).

Half-lives in the mouse were generally of the order of 15 - 20 minutes for cyclohexylalanine-type compounds. This figure was doubled by replacing this group with tertiary butyl. A $t_{1/2}$ of approximately 35 minutes was determined for three such compounds, CT1746, CT1847 and a control compound Ro-31 9790. Most strikingly, oral bioavailability was raised from less than 1% to greater than 30%. The flat oral concentration curve observed in this species suggested that drug was only slowly absorbed. Since the rate of absorption across the G.I. tract is fairly consistent amongst different species and there is no evidence of slow absorption in the rat this may reflect a long residency time in the stomach,

possibly as a result of precipitation. The oral bioavailability of CT1746 and Ro31-9790 was significantly reduced in the rat despite an apparent increase in the systemic half-life to over an hour. This may be interpreted as rapid first pass elimination since gut perfusion studies show that inhibitors are effectively absorbed (Dr. G. Curtiss, personal communication). The aggressive metabolism of the rat might not translate to higher species and so these compounds may be candidates for evaluation in humans.

In summary, this study has extended our knowledge of the mutual activation pathways which may orchestrate the activity of these enzymes in normal physiology and pathology. The measurement of the activity of these enzymes against fluorescent peptide substrates, allowed a range of SARs to be established that culminated in the synthesis of inhibitors with Ki's of less than 10 pM against the target enzyme, gelatinase-A. The discrimination of these inhibitors against collagenase and members of other zinc metalloproteinase families should be reflected in a high tolerance to their presence in the body. At the same time it should be emphasised that a consequence of the activation of MMPs by one another is that even selective inhibitors may have an indirect effect on the activation and hence activity of other MMPs.

This is the first study to report the kinetics of complex formation between a hydroxamic acid based MMP inhibitor and an MMP. The results suggest that they exhibit many of the properties associated with the more potent inhibitors of peptidyl-dipeptidase A.

Finally, the development of methods to measure the *in vivo* activity of these inhibitors has resulted in the identification, of orally bioavailable compounds which may be used to provide treatment of tumour growth and metastasis in man.

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